VOLUME 40 JUNE, 1961 NUMBER 6

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, INC.

Contents

The Nature of the Action of Intravenous Aldosterone: Evidence for a Role of the Hormone in Urinary Dilution. EDMUND H. SONNENBLICK, PAUL J. CANNON and JOHN H. LARAGH 903

Substances Stimulating Glucose Catabolism by the Oxidative Reactions of the Pentose Phosphate Pathway in Human Erythrocytes. ARIEH SZEINBERG and PAUL A. MARKS 914

The Composition and Biosynthesis of Lipids in Human Adipose Tissues. ALFRED GELLHORN and PAUL A. MARKS 925

The Effects of Aging and Degenerative Vascular Disease on the Measurement of Arterial Rigidity in Man. FRANCOIS M. ABBOUD and JOHN H. HUSTON 933

The Synthesis of Heme and Globin in the Maturing Human Erythroid Cell. DAVID G. NATHAN, SERGIO PIOMELLI and FRANK H. GARDNER 940

Quantitative Paper Chromatography of Conjugated 17-Ketosteroids in Plasma. SUZANNE CONRAD, VIRENDRA MAHESH and WALTER HERRMANN 947

Heartburn in Late Pregnancy. Manometric Studies of Esophageal Motor Function. RICHARD NAGLER and HOWARD M. SPIRO 954

The Relationship of Oxygen Cost of Breathing to Respiratory Mechanical Work and Respiratory Force. MAURICE MCGREGOR and MARGARET R. BECKLAKE 971

A Liver Enzyme that Conjugates Sulfobromophthalein Sodium with Glutathione. BURTON COMBES and GENEVA SUE STAKELUM 981

Luekokinetic Studies. IV. The Total Blood, Circulating and Marginal Granulocyte Pools and the Granulocyte Turnover Rate in Normal Subjects. J. W. ATHENS, O. P. HAAB, S. O. RAAB, A. M. MAUER, H. ASHENBRUCKER, G. E. CARTWRIGHT and M. M. WINTROBE 989

Studies of the Rate of Disappearance of Labeled Thyroxine from the Intravascular Compartment. EDWARD J. LENNON, NORMAN H. ENGBRING and WILLIAM W. ENGSTROM 996

Thromboplastin Generation Accelerator, a Newly Recognized Component of the Blood Coagulation Mechanism Present in Excess in Certain Thrombotic States. CHRIS A. PASCUZZI, JOHN A. SPITTEL, JR., JOHN H. THOMPSON, JR. and CHARLES A. OWEN, JR. 1006

Proceedings of the Fifty-Third Annual Meeting of the American Society for Clinical Investigation, Inc. Held in Atlantic City, N. J., May 1, 1961 1019

The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation, Inc., Publication Office, 333 Cedar Street, New Haven 11, Conn.

PHILIP K. BONDY, EDITOR-IN-CHIEF

ASSOCIATE EDITORS

FRANKLIN H. EPSTEIN ALLAN V. N. GOODYER

The Editorial Committee of the American Society for Clinical Investigation, Inc.

ROBERT W. BERLINER, *Bethesda, Md.*
SOLOMON A. BERSON, *New York, N. Y.*
CARLETON B. CHAPMAN, *Dallas, Tex.*
I. S. EDELMAN, *San Francisco, Calif.*
CLEMENT A. FINCH, *Seattle, Wash.*
ALFRED P. FISHMAN, *New York, N. Y.*
DONALD S. FREDRICKSON, *Bethesda, Md.*
ROBERT A. GOOD, *Minneapolis, Minn.*
JOHN B. HICKAM, *Indianapolis, Ind.*
FRANZ J. INGELFINGER, *Boston, Mass.*
HENRY G. KUNKEL, *New York, N. Y.*
MACLYN MCCARTY, *New York, N. Y.*
ARNOLD S. RELMAN, *Boston, Mass.*
JOHN V. TAGGART, *New York, N. Y.*
KENNETH L. ZIERLER, *Baltimore, Md.*

THE JOURNAL OF CLINICAL INVESTIGATION is designed for the publication of original investigations dealing with or bearing on the problems of disease in man, and it is the policy of the editors that the JOURNAL should cover the field of clinical investigations in its broadest sense.

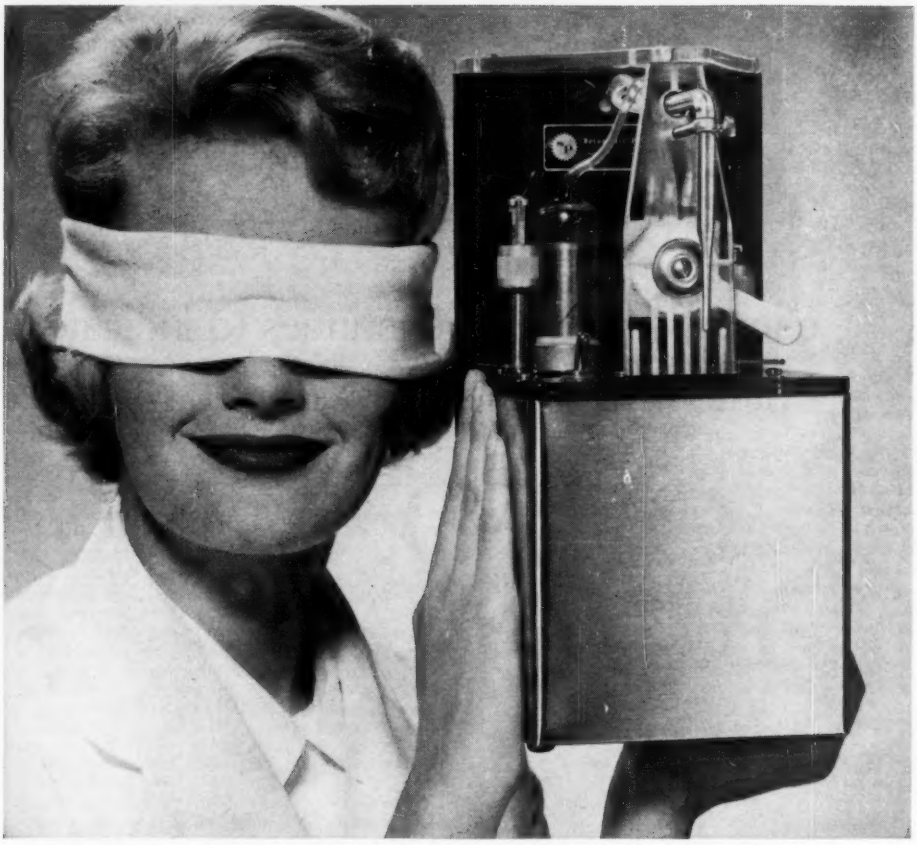
Contributions should be sent to the **Editor-in-Chief, Dr. Philip K. Bondy, 333 Cedar St., New Haven 11, Conn.** In order to hasten the process of reviewing and editing it is requested that each original manuscript be accompanied by a duplicate copy and duplicate illustrations.

In order further to expedite the earliest possible publication of articles submitted to the JOURNAL OF CLINICAL INVESTIGATION they will be published in the order of final processing after review, correcting and editing rather than in the strict sequence of their receipt in this office. Papers which require much editorial change, revision of charts and tables, condensation and rewriting will be delayed according to the time required for their correction. This policy will put a premium on excellence of manuscript as well as the major consideration of content. It is requested that figures be photographed, the photographs accompanying the manuscript. The originals of the figures need not be forwarded except by special request of the editors. For further instructions regarding the preparation of manuscripts, see the first page of the January, 1961, issue.

Each volume will consist of 12 monthly issues appearing throughout the calendar year. The subscription price is $15 per volume, **payable in advance**; foreign countries also $15, payable in U. S. funds. Single copies will be sold when available at $1.50 a copy. Authors may purchase reprints at scheduled prices. **A special subscription rate of $6 per volume of 12 monthly issues is offered to medical students, interns, fellows, and residents of approved hospitals.** Subscriptions are **net postpaid.** Remittance should be made by draft, check or postal money order, payable to **The Journal of Clinical Investigation, 333 Cedar Street, New Haven 11, Conn.** Claims for copies lost in the mails must be received within **thirty** days (domestic), **ninety** days (foreign), of the date of issue. Changes of address must be received at least two weeks in advance of issue.

Second-class postage paid at Lancaster, Pa.

Copyright © 1961, American Society for Clinical Investigation, Inc.



With the S/P Auto Dilutor, you could prepare

repeat-dilutions blindfolded

The S/P Auto Dilutor lets you do a series of identical dilutions faster, easier than ever before. It saves hours of pipetting time and pipette cleaning; eliminates costly glassware breakage. Accuracy better than 1%. Operation is simple: Preset sample and diluent volumes with direct-reading controls . . . *the rest is automatic.* Moving lever upward, draw sample into glass tip. Depress lever, sample is dispensed together with diluent. It's that simple . . . takes just seconds. There's no cleaning required. The sample contacts only the glass tip which is thoroughly diluent-rinsed during each cycle. Put the Auto Dilutor to work in your lab—it's available from S/P exclusively.

No. 67765A—S/P AUTO DILUTOR, 2 ml. to 20 ml. capacity diluent syringe, .01 ml. to 0.1 ml. sample syringe **$250.00**

No. 67765B—S/P AUTO DILUTOR, 2 ml. to 20 ml. capacity diluent syringe, .03 ml. to 0.3 ml. sample syringe **$250.00**

For demonstration or information, contact your S/P Representative . . . or write

scientific products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

GENERAL OFFICES: 1210 LEON PLACE, EVANSTON, ILLINOIS

Regional Offices: Atlanta · Boston · Chicago · Columbus · Dallas · Detroit · Kansas City
Los Angeles · Miami · Minneapolis · New York · San Francisco · Washington

Export Department—Flushing 58, L. I., New York. In Canada: Canadian Laboratory Supplies Limited.
In Mexico: Hoffmann-Pinther & Bosworth, S. A.

clinical experience continues to indicate value of the CYTOTOXIC AGENT...

CYTOXAN®

Cyclophosphamide, Mead Johnson

for palliative chemotherapy of certain types of malignant neoplasms

"Cyclophosphamide [Cytoxan] has proved a valuable addition to chemotherapeutic drugs available for the treatment of malignant diseases of the haemopoietic and reticuloendothelial systems.... Particularly effective in Hodgkin's disease, lymphosarcoma, chronic lymphocytic leukaemia, and myelomatosis...."[1]

"Objective data suggest that this agent [Cytoxan] has advantages not possessed by standard alkylating agents now in clinical use."[2]

"With the use of cyclophosphamide [Cytoxan] there is a relative lack of thrombocytopenia and a diminution in gastrointestinal side-effects, so that it may offer therapeutic advantages over other alkylating agents."[3]

Other Advantages in Clinical Practice: Broad-spectrum application. High therapeutic index. No vesicant activity—may be given orally or parenterally.

(1) Matthias, J. Q.; Misiewicz, J. J., and Scott, R. B.: Brit. M. J. *2*:1837-1840 (Dec. 24) 1960.
(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 o 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

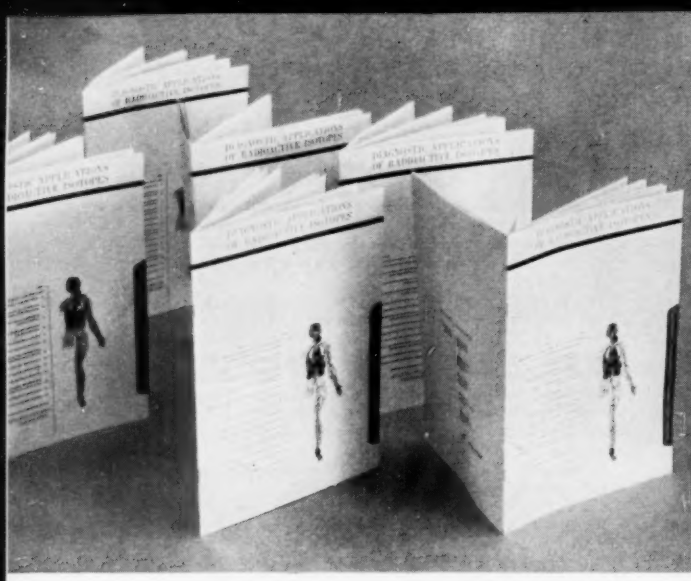
Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.

Mead Johnson
Laboratories

Symbol of service in medicine

52361



Revised edition now available from Nuclear-Chicago

DIAGNOSTIC APPLICATIONS OF RADIOACTIVE ISOTOPES

The newly published second edition of Diagnostic Applications of Radioactive Isotopes contains new radioisotope diagnostic procedures along with modifications of previously presented methods. The contents, listed to the right, suggest the broad scope of this useful and widely accepted publication.

Subject matter describes in outline form the most commonly used procedures for each application, and pertinent references will lead the reader to other methods.

Although the text is intended primarily for those unfamiliar with radioisotope applications to clinical medicine, many will find it a handy refresher guide in this area. Useful also in training programs, the publication provides a convenient reference to commonly used radioisotope diagnostic techniques.

Nuclear-Chicago will be happy to send you a copy without obligation.

nuclear-chicago
CORPORATION

Contents

How to establish your radioisotope program

Evaluation of thyroid function using radioiodine

Estimation of thyroid function with red cell uptake of radioiodine labelled thyronine

Determination of conversion ratio as an estimation of thyroid function

Evaluation of kidney function with radioiodine labelled compounds

Estimation of cardiac output with radioiodinated serum albumin

Estimation of myocardial blood flow

Blood and plasma volume measurements using radioiodinated serum albumin

Measurement of red cell mass with radiochromium

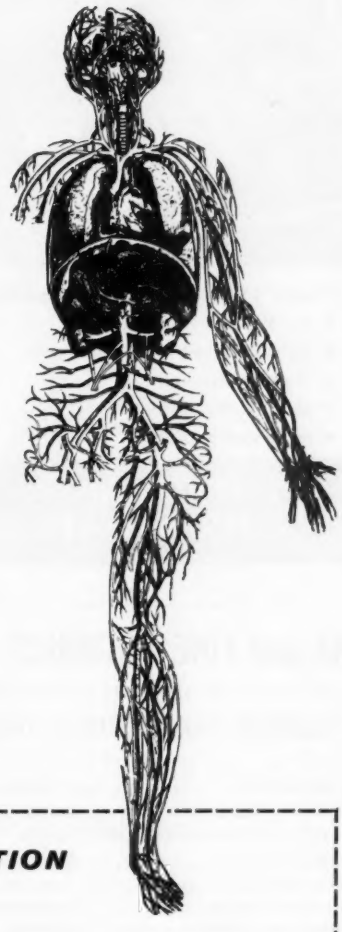
Measurement of red cell survival with radiochromium

Diagnosis of pernicious anemia with radiocobalt vitamin B_{12}

Measurement of fat digestion and absorption with radioiodine labelled triolein and oleic acid

Scanning body areas for radioisotope concentrations

Use of needle-type probe scintillation detectors



NUCLEAR-CHICAGO CORPORATION

329 East Howard Avenue
Des Plaines, Illinois

Please send me the new edition of

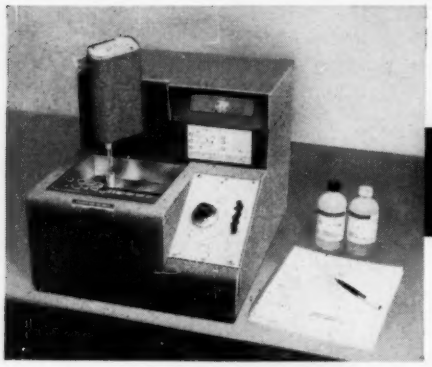
DIAGNOSTIC APPLICATIONS
OF RADIOACTIVE ISOTOPES

Name

Address

City Zone State

first name in Osmometry . . . .



The all-new 1961 Laboratory Model 31L

- smaller
- fully-insulated bath
- 5-year refrigeration
- push button operation
- more stable probes

More laboratories have specified Osmometers from Advanced Instruments than from all other sources combined.

ADVANCED OSMOMETERS

- Regional schools of Osmometry
- Continuing service and complete parts for all earlier models
- Advanced uniform 6-Step instructions for all models

Send for latest specifications today

ADVANCED INSTRUMENTS, INC.

41 Kenneth St., Newton Highlands 61, Mass.
Telephone DEcatur 2-8200

RARE and FINE ORGANICS

A few of the thousands of rare stock chemicals

CETYL PYRIDINIUM CHLORIDE $10.00/100g.

o-AMINO BIPHENYL	$ 9.00/100g.
3,4-DIHYDROXY MANDELIC ACID	45.00/g.
DIISOPROPYL FLUOROPHOSPHATE	16.00/g.
IMIDAZOLE	35.00/KG.
o-IODOBIPHENYL	8.50/10g.
2-KETO GULONIC ACID	30.00/100g.
α-NAPHTHYL ISOTHIOCYANATE	22.50/10g.
o-PHTHALALDEHYDE	21.00/10g.

Write for catalog No. 3 listing 12,000 RARE—

alkaloids	hydrocarbons	steroids
amino acids	keto acids	sugars
enzymes	fatty acids	purines
biochem preps	peptides	carcinogens

Write Dept. "C"

K & K LABORATORIES, INC.

177-10 93rd AVENUE JAMAICA 33, N. Y.

WANTED

Back Issues of

THE JOURNAL OF CLINICAL INVESTIGATION

January, May, June, July, (Pt. I), September, through December, 1955

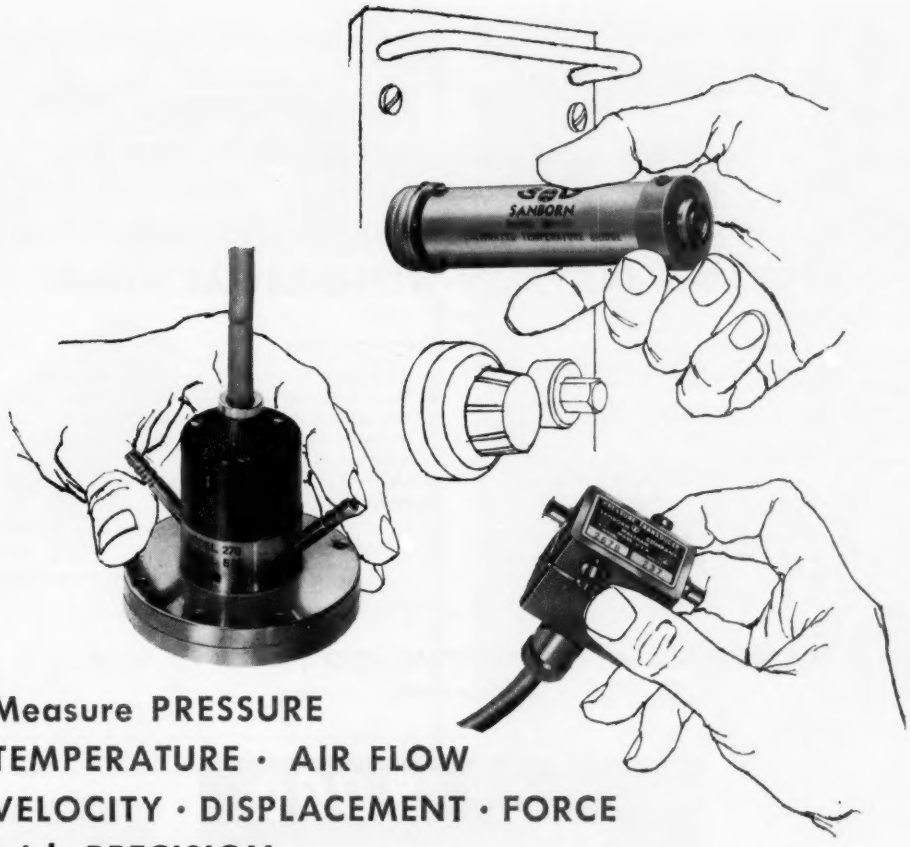
February, July, August, Sept., and November, 1959

February and October, 1960

75¢ per copy and postage

For particulars about mailing write to

The Journal of Clinical Investigation
Business Office
333 Cedar Street
New Haven 11, Conn.



Measure PRESSURE TEMPERATURE · AIR FLOW VELOCITY · DISPLACEMENT · FORCE with PRECISION SANBORN TRANSDUCERS

You can meet a wide variety of requirements from this broad selection of accurate, compact Sanborn Transducers. Series 267 and 268 Physiological Pressure Transducers are designed for either differential or single-ended measurements in such applications as cardiac catheterization and studies of circulatory, respiratory, esophogeal, spinal or gastric pressures. Two basic sensitivities are available: 1.0 or 0.1 mm Hg produces 1 cm. chart deflection. Model 270 Bi-Directional Differential Gas Pressure Transducer permits measurements of small pressure changes (1 cm/0.5 mm H_2O) over a wide range with excellent linearity and low drift. Pneumotach heads for respiratory air flow measurements are available for use with the 270. Excellent stability and temperature compensation also make the Model 270 particularly suitable for plethysmography applications.

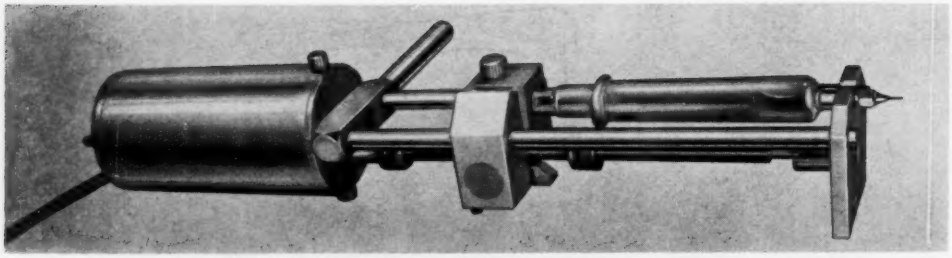
Model 760-53 Calibrated Temperature Bridge and a variety of interchangeable thermistor probes are available for use with any Sanborn Carrier Preamplifier for accurate recording or monitoring of physiological temperatures with full scale sensitivity of 1°C and 2.5°C.

In addition, Sanborn offers pneumograph and pulse wave attachments, heart sound microphones, linear velocity and displacement transducers, and transducers for force measurements in myographic studies. For complete information contact your nearest Sanborn Branch Office or Service Agency — or write Manager, Research Instrument Sales, Medical Division.

MEDICAL DIVISION

SANBORN COMPANY

175 Wyman St., Waltham 54, Massachusetts



PORTABLE INFUSION-WITHDRAWAL PUMP

Catalog No. 1100

Small size and complete portability permit use in limited working space where flexibility of mounting is important. Pumping mechanism consists of a precision-cut, stainless steel lead screw and moving carriage assembly. Limit stops automatically terminate pumping action at any pre-set point. Its special construction allows the pump to be placed in any position without impairing pumping action.

The pump is designed to use interchangeable, plug-in motors which are synchronous and reversible. A wide variety of motors is available to produce any desired pumping rate. Motors are quickly interchanged by the user with no tools required.

SPECIFICATIONS

- Rates from 10 ml./min. to 0.00096 ml./min.
- Max. reproducibility due to synchronous motors
- 110–120 volt A.C., 60 cycle operation
- Accepts standard Luer Lok syringes
- Noiseless in operation
- Size: 15″ × 3″ × 2″, exclusive of handle
- Weight: under 4 pounds

Pump complete with 1 motor $135.00
Extra motors, each ..... $ 12.00
Prices are f.o.b. Dover, Mass.

Data Sheet 1100 and Catalog 1960–61 available on request

HARVARD APPARATUS CO., INC. • Dover, Mass., U. S. A.
(a non-profit organization)

NOW OVER **18,000** **BIOS** CHEMICALS

- Nitrourea
- Nonamethylenediamine
- Octadecanedicarboxylic Acid
- Opianic Acid
- Oxamic Acid
- Oxindole
- Palmitoleic Acid
- Penicillamine Hydrochloride
- Pentachloroacetone
- Pentamethonium Bromide
- Pentamidine
- Phenanthridine
- Phenazine Methochloride
- Phenolphthalein Phosphate (Sodium Salt)
- Phenoltetrachlorophthalein
- Phenolthymolphthalein
- 9-Phenylacridine
- 2-Phenylazo-p-cresol

Ask for our new complete catalogue

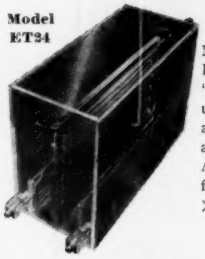
BIOS *Laboratories, Inc.*

17 West 60th St., New York 23, New York
PLaza 7-8171

low cost-

HIGH VOLTAGE PAPER ELECTROPHORESIS TANKS & POWER SUPPLIES

Model ET24

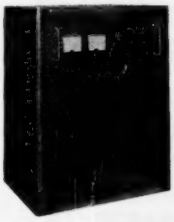


Model ET24 High Voltage Paper Electrophoresis Tank for "fingerprinting" peptides; also useful for separating amino acids, nucleotides, carbohydrates and other charged particles. Accepts standard size Whatman filter paper measuring 18¼ × 22½ inches.

Model ET48 Electrophoresis Tank is a larger tank for separating out all of the amino acids and will accept up to a 48" length sheet (20" wide). Basic tank can be easily adapted for various rack configurations.

Superior features of Models ET24 and ET48

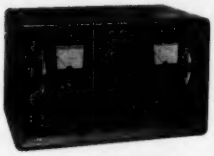
- Transparent lucite allows the researcher to see what is going on inside the tank.
- The tanks can be placed on bench tops and inside hoods.
- Other types of racks easily adaptable to basic tanks.
- Safety interlock switches on all tanks.
- Drain plugs at side of tank.



Model EHV2000 "Electrophoresis" High Voltage Power Supply will deliver up to 2000 volts at 150 milliamps.

The Model EHV5000 Power Supply will deliver up to 5000 volts at 300 milliamps.

Model EHV5000



Model EHV2000

Superior features of Models EHV2000 and EHV5000

- Solid state circuitry throughout.
- Instant starting.
- No warmup.
- No vacuum tubes.
- Overload circuit with automatic cutout for maximum safety to personnel.

Complete literature upon request.

SERVONUCLEAR CORP.
28-21 Astoria Blvd., Long Island City 2, N. Y.
Yellowstone 2-3353

did you know you can

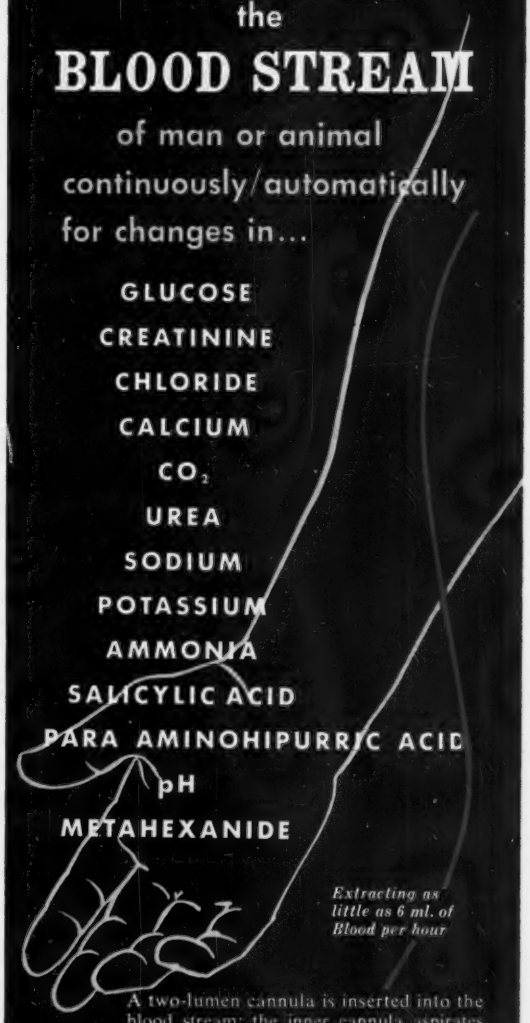
MONITOR

the

BLOOD STREAM

of man or animal continuously/automatically for changes in...

GLUCOSE
CREATININE
CHLORIDE
CALCIUM
CO_2
UREA
SODIUM
POTASSIUM
AMMONIA
SALICYLIC ACID
PARA AMINOHIPURRIC ACID
pH
METAHEXANIDE



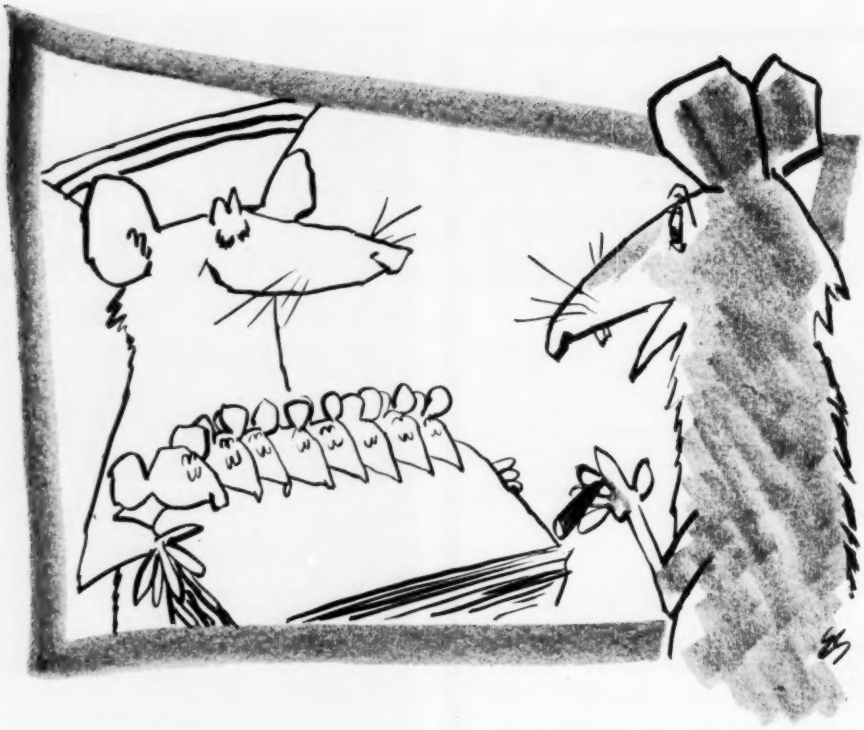
Extracting as little as 6 ml. of Blood per hour

A two-lumen cannula is inserted into the blood stream; the inner cannula aspirates blood while the outer cannula continuously feeds heparin into the blood aspirated, without letting heparin into the blood stream...

with the TECHNICON® AutoAnalyzer®

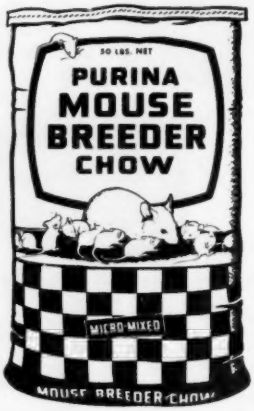
Write and we will forward reprints of published papers.

TECHNICON INSTRUMENTS CORP.
22 RESEARCH PARK • CHAUNCEY, NEW YORK



"only 8?!!... we've got to get mouse breeder chow!"

Purina Mouse Breeder Chow was developed especially for mouse breeder colonies. In our own laboratories, this improved ration increased average litter size to 12 young per litter (ICR strain). Offspring may be weaned at an earlier age (15 to 18 days) permitting the female a longer recovery period before the next litter. Ingredients are laboratory controlled to assure uniform nutrition and constant high quality. Get fresh supplies in any desired quantity by calling your local Purina Dealer. Or write H. A. Graff, Ralston Purina Company, St. Louis 2, Missouri.



PURINA LABORATORY CHOWS

5 special rations for all laboratory animals

Leukemia.....

'LEUKERAN'® brand

CHLORAMBUCIL (formerly known as C. B. 1348)

FOR CHRONIC LYMPHOCYTIC LEUKEMIA

A derivative of nitrogen mustard, it has provided amelioration of follicular lymphoma, lymphocytic lymphoma with or without leukemia, and Hodgkin's disease.

Sugar-coated Tablets of 2 mg.

'MYLERAN'® brand

BUSULFAN

FOR CHRONIC MYELOCYTIC LEUKEMIA

'Myleran' has been reported to induce remissions, lasting up to two years, in chronic myelocytic leukemia. In addition to the decrease in total white cell count and a selective reduction of immature myeloid cells, it usually gives, early after its administration, a rise in hemoglobin level and pronounced subjective improvement.

Tablets of 2 mg.

'PURINETHOL'® brand

MERCAPTOPURINE

FOR ACUTE LEUKEMIA AND CHRONIC MYELOCYTIC LEUKEMIA

'Purinethol' provides worth-while temporary remissions, either partial or complete, in a high percentage of patients. In general, a higher proportion of children than adults with acute leukemia respond favorably.

Tablets of 50 mg.

Facilities for complete and frequent blood counts must be available for patients receiving 'Leukeran', 'Myleran' or 'Purinethol'.

Full information about these products will be sent on request.

BURROUGHS WELLCOME & CO. (U.S.A.) INC., Tuckahoe, New York

The MIKROTIT, microtitrater, manual spiral-loop dilution technique simplifies and accelerates serological titrations.

6 loops can be handled at one time

RAPID ACCURATE
A MUST FOR VIROLOGY AND SEROLOGY

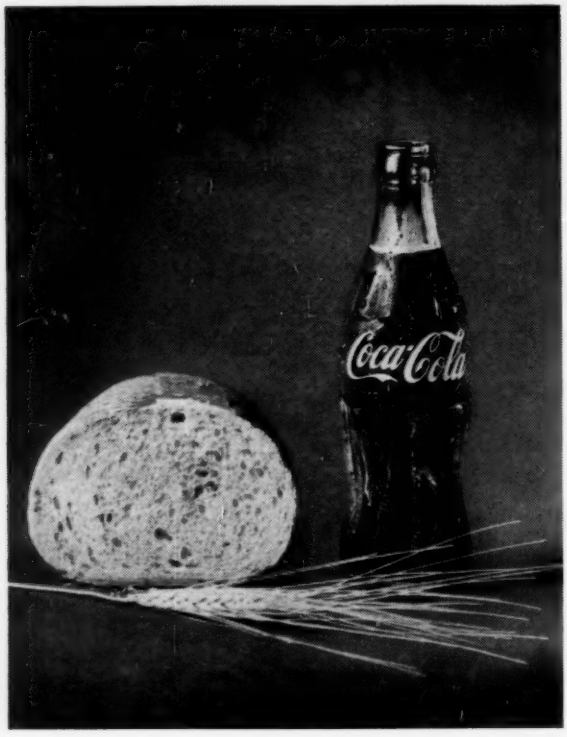
now in use in most leading laboratories
Serial Dilutions can be rapidly done using 1/10th the reagent of the older pipette techniques

MIKROTIT

Write for additional information

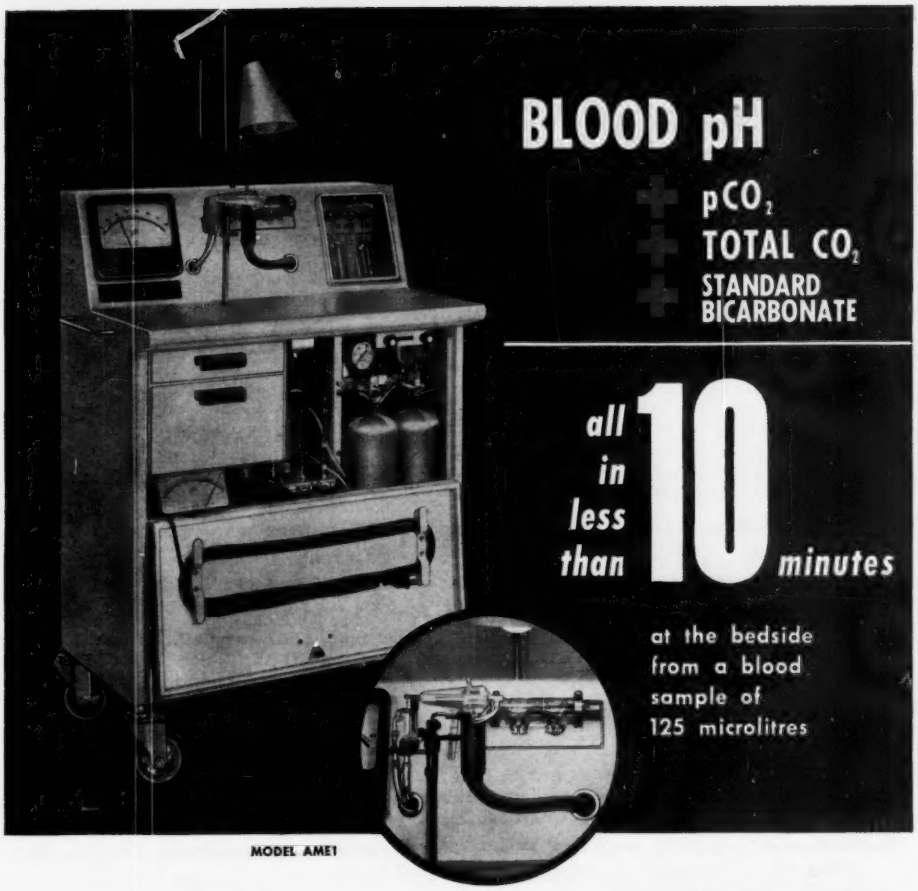
INTERCONTINENTAL SCIENTIFIC CORP.

23 West 60th Street New York 23, N.Y.



Coca-Cola, too, has its place in a well balanced diet. As a pure, wholesome drink, it provides a bit of quick energy.. brings you back refreshed after work or play. It contributes to good health by providing a pleasurable moment's pause from the pace of a busy day.

DRINK
Coca-Cola
TRADE-MARK ®


BLOOD pH
+ pCO_2
+ TOTAL CO_2
+ STANDARD BICARBONATE
all in less than 10 minutes
at the bedside from a blood sample of 125 microlitres

MODEL AME1

Astrup *Micro* Apparatus *by* RADIOMETER

The increasing importance being attached to the acid base balance in blood, both in the clinical and research fields, has raised a major requirement for a new approach to these measurements.

Such determinations must be made swiftly, and accurately, with minimum sample requirements, not only for routine clinical use but for the hitherto unserved areas of pediatrics, anesthesiology, and small animal research.

The Ultra Micro Astrup Apparatus by Radiometer fills such a need. A complete acid-base determination covering pH, pCO_2, total CO_2, Standard Bicarbonate, excess acid or base, and Buffer Base (according to Singer and Hastings), can be made **at the bedside** in less than 10 minutes, on a capillary blood sample of 100-125 ul.

Mounted on a wheeled cabinet, the unit is completely self contained except for plug-in connection to the power mains.

Write for further details on the AME1 and the Astrup Method.

(Also available separately is the thermostated Micro Capillary Electrode Set E5020, for blood pH measurements).

SOLD AND SERVICED IN U.S.A. BY
THE LONDON COMPANY
(Formerly Welwyn International Inc.)
3355 Edgecliff Terrace CLEVELAND 11 OHIO

RADIOMETER
72 Emdrupvej COPENHAGEN, DENMARK

In Canada Contact any Branch of Canadian Laboratory Supplies Limited

REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

June, 1961

Vol. VI, No. 6

Editorial

Les Foetopathies. D. ALAGILLE.

Original Articles

La Reaction de Fixation au Latex (F II L.P.) dans les Applications Rhumatismales et non Rhumatismales. G. H. FALLET, E. MEYER et J.-J. SCHEIDEGGER.

Etude du Mecanisme d'Action de l'Hydrochlorothiazide dans l'Hypertension Arterielle. Role de l'Hypokaliemie. D. FRITEL, J. QUICHAUD, M. HODARA et J. TRUFFERT.

Porphyrie Experimentale Observee Chez le Rat Blanc a la Suite de l'Intoxication par l'Hexachlorobenzene. A. GAJDOS et M. GAJDOS-TORÖK.

Action Therapeutique de l'Acide Adenosine-5-Monophsophorique sur la Porphyrie Experimentale du Rat Blanc due a l'Intoxication par l'Hexachlorobenzene. A. GAJDOS et M. GAJDOS-TORÖK.

Original Notes

Quelques Modalites de l'Activite des Muscles Respiratoires chez le Parkinsonien. L. DELHEZ et J. M. PETIT.

Le Transfert du Na^{24} et du K^{42} du Sang dans la Bile chez le Cobaye au Cours de la Cholerese a Debit Bloque (Stop Flow Analysis). J. CHENDEROVITCH, S. TROUPEL, H. RENAULT et J. CAROLI.

Modifications du Taux de Na, de Cl, de K, et de H_2O au Niveau des Reins, Consecutives a l'Application du Salyrgan. R. DZURIK, D. HOSTYNOVA et B. KRAJCI-LAZARY.

Essai d'Interpretation Quantitative des Qualites des Reactifs Anti-Gm. C. ROPARTZ, P. Y. ROUSSEAU et L. RIVAT.

Influence de la Somatotrophine Hypophysaire sur les Activites Enzymatiques des Globules Rouges de Souris *in vivo*. G. BROUN.

Note sur un Nouveau Mode de Detection des Glucuronides de Steroides en Chromatographie sur Papier. O. CRÉPY, B. LACHÈSE et O. JUDAS.

General Review

Les Antigenes de Transplantation. A. CASTERMANS.

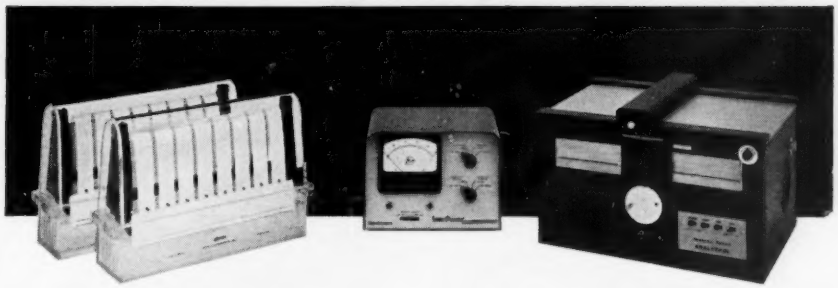
Technical Note

Preparation et Utilisation des Anticorps Fluorescents. A. PELTIER et P. BURTIN.

Subscription rate, $19.00

Special rate for interns and residents, $13.00

EDITIONS MEDICALES FLAMMARION, 22, rue de Vaugirard, PARIS 6ème. France.



Durrum-type cell: Holds eight filter strips on folding rack in inverted V position.

Duostat: Provides regulated power for single-cell or two-cell operation.

Analytrol: Scans and quantitates dyed paper strips.

Auxiliary equipment set: Provides all the accessories needed for serum protein analysis.

A complete system for

PAPER ELECTROPHORESIS

If analysis by electrophoresis is missing from your lineup of services, why not take a few minutes to inquire about the Beckman Model R Paper Electrophoresis System. In laboratories using this important diagnostic aid, Model R is the instrumentation of choice.

1. *It is a complete system:* The three basic instruments and accessories which make up Model R provide all the equipment needed to do a wide variety of electrophoretic separations.

2. *It operates simply, dependably:* Straightforward procedures make it easy for technicians to work with the System.

3. *It is versatile:* In addition to serum proteins, the System can separate hemoglobins, serum lipoproteins, serum glycoproteins and other biochemical materials.

4. *Results are reproducible:* Equipment keeps variables under control, maintains uniform conditions from test to test.

Because the Model R System is the standard in the field, results can be compared with those from other laboratories and with published reports in the literature. For further information, please fill in and return the coupon below.

Beckman / **Spinco Division**
Beckman Instruments, Inc.

Spinco Division, Stanford Industrial Park, Palo Alto 16, California
Please send me further information on the Beckman Model R System and a reprint of "The Clinical Significance of Paper Electrophoresis."

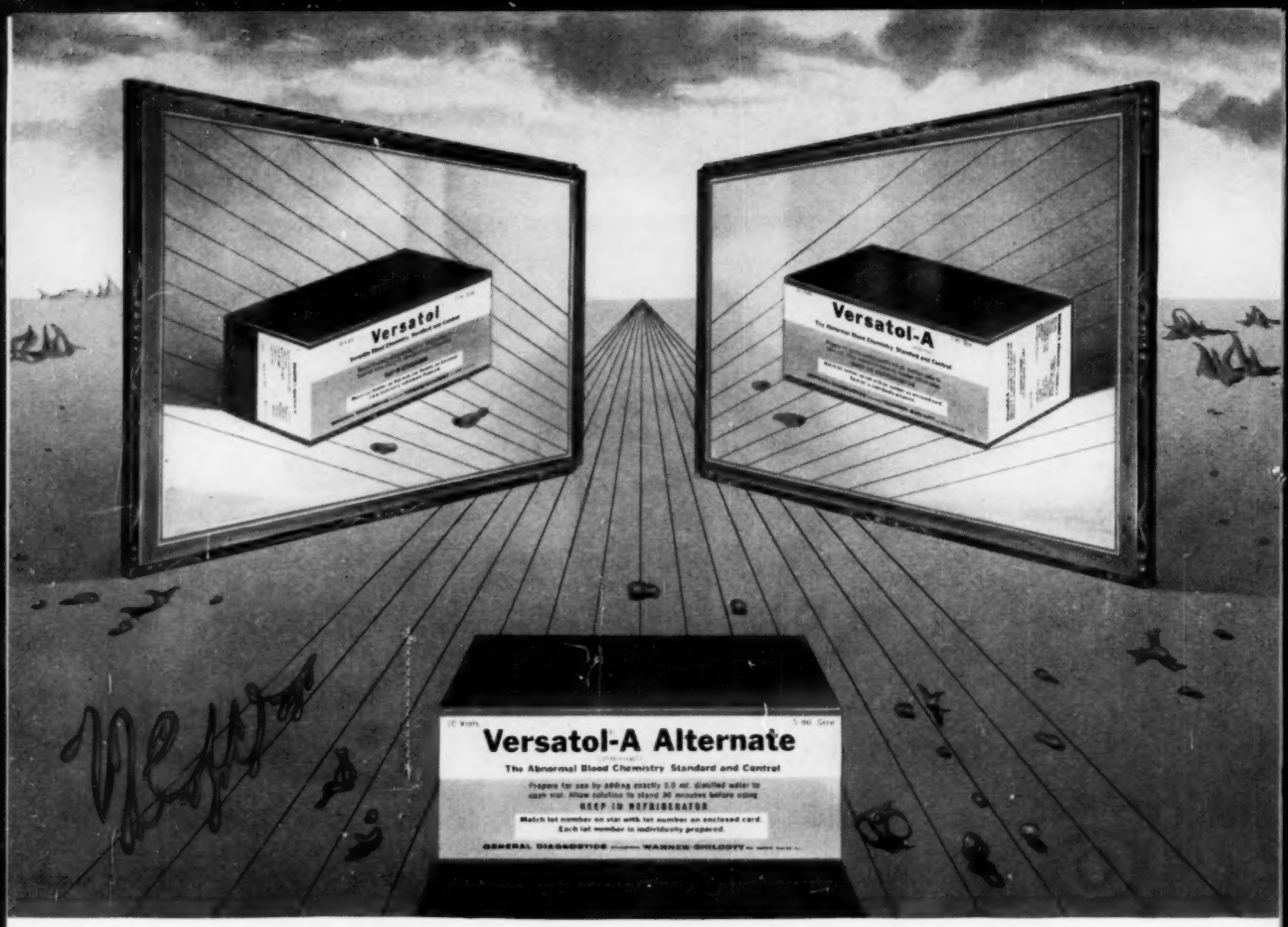
name _____

institution _____

address _____

_____

S-73C2



Reflections of a Blood Chemistry Standard

To reflect without distortion chemical changes in the patient, a new standard-in-serum is now available to extend the range of the Versatol series.

VERSATOL-A ALTERNATE, the new product for the quality clinical laboratory, has each of 16 constituents standardized in the abnormal range.

VERSATOL-A ALTERNATE is essentially a mirror image of Versatol-A. VERSATOL-A ALTERNATE provides blood chemistry values constructed for clinically significant pathological areas different from those in VERSATOL-A.

For example, the PBI in Versatol-A is abnormally low, the area which so often proves troublesome. In VERSATOL-A ALTERNATE the PBI is abnormally high—because so many have asked for an abnormally high reference point to use in calibration—and, of course, as a control for elevated PBI specimens.

Of course, Versatol is the ideal *normal* serum standard and control. All three standards derive from a human serum base—with specific constituents selectively dialyzed out—or reduced to a constant level. Their known absolute values are actual weigh-in values, as is true of any primary standard.

Remember: In the Versatol series you have the *quality* standard and control serum products at *routine* control prices.

Initially, VERSATOL-A ALTERNATE is available in boxes of 10 vials each. Reconstitution with 5 ml. of distilled water provides a 5.3 ml. of standard solution per vial. Cost to the laboratory, like Versatol and Versatol-A 10's, is $21.00 per box, or $0.39 per ml.

Place your order today for VERSATOL-A ALTERNATE and the other products in the Versatol series.

GENERAL DIAGNOSTICS DIVISION **WARNER-CHILCOTT** DIV. MORRIS PLAINS, N.J.

THE NATURE OF THE ACTION OF INTRAVENOUS ALDOSTERONE: EVIDENCE FOR A ROLE OF THE HORMONE IN URINARY DILUTION *

By EDMUND H. SONNENBLICK,† PAUL J. CANNON AND JOHN H. LARAGH

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York, N. Y.)

(Submitted for publication September 15, 1960; accepted January 13, 1961)

Aldosterone increases the renal retention of sodium chloride and promotes the excretion of potassium ions (1–11); but where and how the hormone acts in the renal tubule is poorly understood. The present study was designed to characterize further the mode and site of action of aldosterone in the nephron.

Information can be obtained about the site of action of a compound by observing its effect on free water formation (12). According to a current concept of renal physiology (13), reabsorption of solute (sodium chloride) is isosmotic in the proximal tubule (14), whereas, in the more distal segments, reabsorption may or may not be isosmotic (15, 16). During water diuresis (absence of antidiuretic hormone) the distal reabsorption of solute is selective, i.e., occurs without isosmotic amounts of water, leaving "solute-free" water behind for excretion (17). Thus, by determining the effect of a compound on solute excretion and on free water formation during maintained water diuresis, information may be obtained about its site of action (12). If aldosterone were to act to promote isosmotic reabsorption of sodium, the consequent reduction in solute excretion would be accompanied by a fall in urine flow and no change or fall in free water excretion. However, if aldosterone acts only at a more distal site, where reabsorption is selective, the reduced sodium excretion would be accompanied by no change in urine flow and hence, by a rise in free water excretion.

In the present study it has been shown that aldosterone can promote abstraction of sodium chloride exclusive of water from the tubular urine. This action takes place in the tubule at a locus distal to that of isosmotic reabsorption. Also, this effect of the hormone on sodium chloride reabsorption appears to be dissociated from the effect on potassium excretion.

EXPERIMENTAL

Seven studies were performed on 3 subjects without evidence of renal or cardiovascular disease. Four of the experiments were done with subjects maintained on a normal NaCl intake (4 to 6 g NaCl per day for 7 days), two with the subjects on a higher intake of salt for 7 days (8 to 20 g NaCl per day), and one after drastic salt deprivation (less than 250 mg per day for 6 days). In two subjects on normal salt intake, control studies of similar protocol but without administration of aldosterone were performed. All experiments were conducted in the fasting state at the same early morning hour with the subjects in a recumbent position.

Water diuresis was induced by having the patient ingest about 1,500 ml of water by mouth during the hour before the experiment and was subsequently maintained by intravenous infusion of 5 per cent dextrose and water at a constant rate, at least 2 ml per minute greater than urinary output. Adequate control periods were obtained until a steady state of maximum urine flow was demonstrated, so that any subsequent change in free water clearance could not be attributed to an increasing water diuresis.

When a steady state of maximum water diuresis had been achieved, 1 mg of *d,l*-aldosterone monoacetate in 10 ml of 10 per cent ethanol was administered intravenously.[1] This was followed by another 1 mg given in the dextrose and water infusion over the next hour. After the aldosterone administration was complete, the infusion of dextrose and water was continued at a constant rate.

Blood samples were taken via an indwelling heparinized needle and urine collections were made with an indwelling catheter except in Subjects S.H. and M.U., young males in whom adequate voluntary bladder emptying was possible at high rates of urine flow.

* This work was supported by the United States Public Health Service (Grant H-1275) and by Mrs. R. C. duPont.

† Present address: National Heart Institute, Bethesda, Md.

[1] *d,l*-Aldosterone monoacetate, 1,000 μg equivalent to 500 μg active *d*-aldosterone supplied by Ciba Pharmaceutical Products, Summit, N. J.

TABLE I

Detailed protocol of a typical experiment *

Time	C_{PAH}	C_{In}	V	U_{osm}	C_{osm}	C_{H_2O}	$U_{Na}V$	$U_{Cl}V$	$U_{K}V$	pH	Plasma Osm	Plasma Na	Plasma K	Plasma Cl
min	*ml/min*	*ml/min*	*ml/min*	*μOsm/ml*	*ml/min*	*ml/min*	*μEq/min*	*μEq/min*	*μEq/min*		*μOsm/ml*	*μEq/ml*	*μEq/ml*	*μEq/ml*
−150	2,000 ml H_2O p.o.													
−113											283	136	4.7	97
−110	Inulin and PAH prime and start infusion of inulin and PAH in 5% dextrose and water at 20 ml/min.													
−78 to −29	635	148	18.3	54	3.5	14.7	135	100	35	5.65	281	136	3.8	98
−29 to −8	612	131	18.5	52	3.4	15.0	150	91	54	5.8				
0	1 mg *d,l*-Aldosterone given intravenously and 1 mg added to 1L of dextrose and water infusion.													
−8 to +21	637	137	17.3	46	2.9	14.5	163	118	80	5.75	273	134	3.6	97
+21 to +50	657	148	17.8	35	2.2	15.6	157	110	69					
+50	Aldosterone infusion completed; dextrose and water continued at 20 ml/min.													
+50 to +80	640	151	18.5	33	2.2	16.3	109	70	104	5.75	272	131	3.6	96
+80 to +110	646	141	18.0	31	2.0	16.0	83	54	86	5.5				
+110 to +139	633	155	18.5	30	2.0	16.5	48	46	61	5.3				
+139 to +168			17.9	30	2.0	15.9	57	47	77	5.4	270	128	3.7	93
+168 to +200	633	140	17.2	26	1.6	15.6	43	43	65	5.35				
+200 to +262			18.4	19	1.3	17.1	70	61	57		268	128	3.7	94
+212	Dextrose and water infusion stopped.													
+262 to +322			18.4	20	1.4	17.0	52	75	35					
+322 to +367			19.2	18	1.3	17.9	63	77	60					
+367 to +442			18.9	22	1.5	17.4	64	77	28					
+442 to +487			18.7	26	1.8	16.6	101	62	32					
+487 to +577			18.3	40	2.7	15.6	157	46	48					
+577 to +650			18.2	46	3.1	15.1	142	64	58					

* Subject S.H., 72.5 kg male on constant 6 g NaCl intake; 2,000 ml H_2O given p.o. at 6 a.m.

Plasma and urine samples were analyzed for inulin or creatinine, para-aminohippuric acid, and sodium, potassium and chloride, by methods previously described (12). The urinary pH was measured by a Beckman pH meter on freshly voided samples and the urine and plasma urea concentrations were determined by a modification of the urease method of Van Slyke and Cullen (18). The urine and plasma total solute concentration was measured with a Fiske osmometer.

Calculations. As indicated by Smith (13) and by Wesson and Anslow (17), urine can be divided into two moieties: the osmolar clearance (C_{osm}), the volume of water necessary to contain the urinary solutes in a solution isosmotic with the plasma ($C_{osm} = UV_{osm}/P_{osm}$); and the free water clearance (C_{H_2O}), the net excess or deficit of water beyond the osmolar clearance. During water diuresis the free water clearance is a positive value and is calculated as $C_{H_2O} = V - U_{osm}V/P_{osm}$, where V is the urine flow in milliliters per minute and U_{osm} and P_{osm} are the solute concentrations of urine and of plasma in milliosmoles per kilogram of water.

RESULTS

The results of these experiments are summarized in Tables I, II and III and in Figures 1 through 4. In Table III, a typical control protocol is given. In Table I the complete protocol of one experiment is given, and in Figure 1 the com-

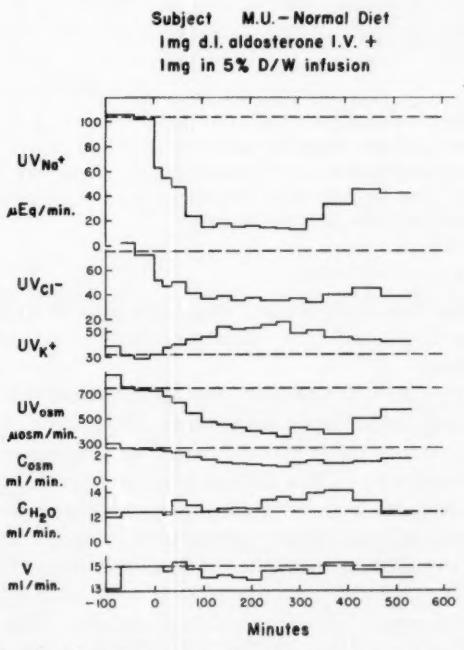


FIG. 1. GRAPH OF THE RESULTS OF A REPRESENTATIVE CLEARANCE STUDY (SEE TEXT).

TABLE II

Effect of intravenous aldosterone on electrolyte, solute and water excretion during maintained water diuresis *

Patient	Period	GFR	RPF	$U_{Na}V$	U_KV	$U_{Cl}V$	pH	P_{osm}	U_{osm}	V	C_{osm}	C_{H_2O}
		ml/min	*ml/min*	*μEq/min*	*μEq/min*	*μEq/min*		*μOsm/ml*	*μOsm/ml*	*ml/min*	*ml/min*	*ml/min*
S.H. (normal diet)	Control	C_{In} 139	C_{PAH} 623	150	54	91	5.80	281	52	18.5	3.4	15.0
	Aldo	C_{In} 140	C_{PAH} 633	52	35	75	5.35	268	19	18.4	1.4	17.0
M.U. (normal diet)	Control			104	30	77.3	6.40	290	50	15.0	2.6	12.4
	Aldo			14	53	36.4	6.10	286	26	14.9	1.3	13.6
M.U. (8 g NaCl in daily diet)	Control	C_{In} 114	C_{PAH} 620	91	39	33		271	46	13.9	2.4	11.5
	Aldo	C_{In} 115	C_{PAH} 573	32	60	19		270	23	14.0	1.2	12.8
J.O. (low NaCl diet)	Control	C_{Cr} 196		257	131	288		291	45	33.5	5.1	28.3
	Aldo	C_{Cr} 200		156	110	188		282	18	31.3	2.0	29.4
J.O. (8 g NaCl in daily diet)	Control	C_{Cr} 204		714	67	1,155		277	89	45.0	14.8	31.0
	Aldo	C_{Cr} 200		346	84	498		273	39	41.2	5.9	35.3
J.O. (20 g NaCl in daily diet)	Control	C_{In} 213		753	87	711	6.53	288	75	41.4	10.8	31.1
	Aldo	C_{In} 211		312	117	322	6.10	278	30	36.8	4.0	32.8
M.U. (low NaCl diet)	Control	C_{In} 101	C_{PAH} 640	6.5	70	3.4		267	54	10.1	2.1	8.0
	Aldo	C_{In} 116	C_{PAH} 703	5	65	3.8		258	44	10.1	1.7	8.4

* Plotted from control periods at peak of water diuresis and from an average of two or three consecutive experimental periods taken at the observed peak effect of aldosterone on solute excretion. The corresponding electrolyte and hemodynamic values for these selected periods are given.

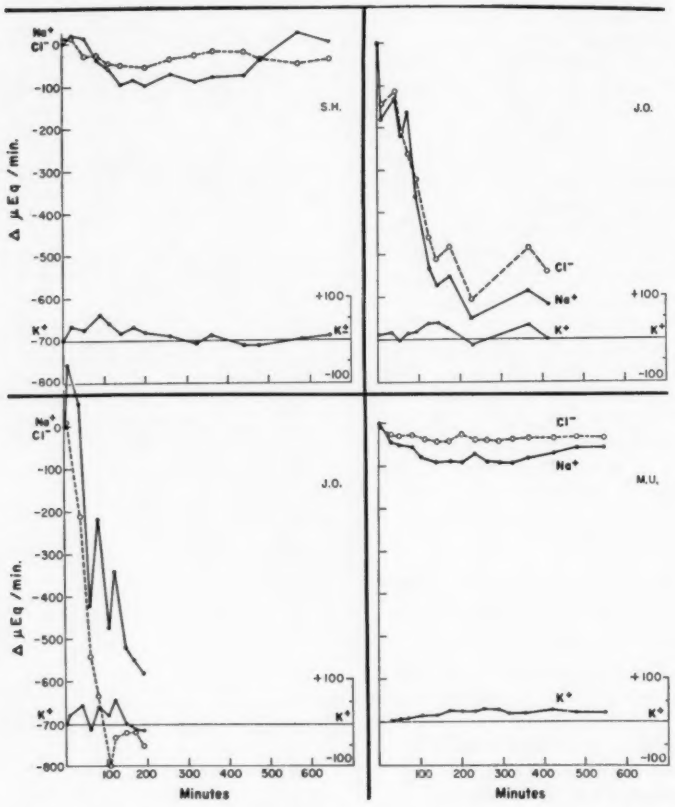


FIG. 2. EFFECT OF ALDOSTERONE ON SODIUM, POTASSIUM AND CHLORIDE EXCRETION PLOTTED AGAINST TIME IN FOUR DIFFERENT STUDIES. On the ordinates the net changes from the control values are plotted for each collection period. The increased potassium excretion is often out of phase with the reduction in sodium chloride excretion. Also the increase in potassium excretion is of considerably lesser magnitude than the reduction in sodium chloride excretion (see text).

plete results of another experiment are depicted graphically.

Effect on renal hemodynamics. Aldosterone administration produced no significant changes in either glomerular filtration rate or renal plasma flow (Tables I and II). The subjects varied considerably in their control glomerular filtration rate values from 102 ml per minute in M.U. to 213 ml per minute in J.O.; a similar variation was noted in the renal plasma flow. Because no consistent alteration was observed in a given patient in these values after injection of the hormone, the marked changes in solute and electrolyte excretion to be described were not attributable to alterations in renal hemodynamics and therefore appeared to be due to the action of aldosterone on the renal tubules.

Effect on sodium and chloride excretion. Following aldosterone administration there was a delay of from 20 to 60 minutes before a significant reduction in sodium chloride excretion developed. During the first 20 to 60 minutes of aldosterone administration variable effects were observed, and in two of the seven studies a transient increase in sodium chloride excretion actually occurred in this early period (Figure 2, Tables I and II). Then, sodium chloride excretion was sharply reduced. The reduction reached a maximum 2 to 4 hours

after the aldosterone administration was begun. The depressed sodium chloride excretion did not return toward the control values until 6 to 8 hours after completion of aldosterone administration.

The magnitude of the sodium retention (i.e., $\Delta U_{Na}V$ from the control level) was the greatest in Subject J.O., who had the highest filtered load of sodium, and was least in Subject M.U. when he was sodium-depleted. Presumably, in this latter instance a maximal endogenous aldosterone effect was present (19, 20), and no further reduction in the $U_{Na}V$ could be produced by aldosterone administration. In the other six studies the rate of sodium excretion was reduced by from 40 to 86.5 per cent of the control values, and the absolute rate of excretion was reduced by from 59 to 441 μEq per minute in these six studies.

A parallel but, in general, lesser reduction in chloride excretion was observed. In the first six of the seven experiments (Tables I and II), chloride excretion was reduced by from 21 to 66 per cent of the control values and the decrements in rate of chloride excretion ranged from 14 to 657 μEq per minute in these six experiments. In one experiment (J.O. on 20 g NaCl intake), the fall in chloride excretion after aldosterone actually exceeded the fall in sodium output by a considerable degree. The reason for this single observation remains obscure.

That the changes observed in sodium and chloride excretion were due to aldosterone and not to prolonged water diuresis is evident from data obtained in two control studies. Water diuresis was maintained for about 6 hours in two recumbent normal subjects. No significant changes in sodium, potassium or chloride excretion were observed during this period. In Subject O.G., whose protocol is presented in Table III, a stable water diuresis was established. Osmolar clearance fell slightly in the first 2 hours with stabilization at 2.7 ml per minute while urine volume and free water clearance (C_{H_2O}) remained relatively unchanged. In a second normal subject, E.S., a similar course of results prevailed with an average urine flow of 17.6 ml per minute in the first 190 minutes and 16.0 ml per minute in the ensuing 160 minutes. Osmolar clearance fell slightly from an average of 2.8 to 2.5 ml per minute during the same periods with a concomitant decline of free water clearance from 14.8 to 13.5 ml per minute. Average rates of electrolyte excretion in the first 190 minutes were: Na^+, 324; K^+, 106; and Cl^-, 424 μEq per minute; in the latter 160 minutes: Na^+, 411; K^+, 70; and Cl^-, 355 μEq per minute. These control studies are in agreement with the previous work of other investigators (21–23). Altogether, these results appear to exclude the possibility that the observed alterations in electrolyte excretion after aldosterone were simply a consequence of prolonged water diuresis.

Effect on potassium excretion and on urinary pH. In every experiment but one (M.U., salt-depleted) potassium excretion was at least transiently increased. The increased potassium excretion often preceded the sodium chloride retention

TABLE III

Detailed protocol of a typical control experiment *

Time	V	U_{osm}	C_{osm}	C_{H_2O}	$U_{Na}V$	$U_{Cl}C$	U_KV	Plasma Osm	Plasma Na	Plasma K	Plasma Cl
min	*ml/min*	*μOsm/ml*	*ml/min*	*ml/min*	*μEq/min*	*μEq/min*	*μEq/min*	*μOsm/ml*	*μEq/ml*	*μEq/ml*	*μEq/ml*
0	1,500 ml H_2O p.o., 30 minutes before start of infusion to recumbent subject. 5% Dextrose and water infusion at 18 ml/min.										
+23 to 67	17.4	47	2.9	14.5	299	269	29	287	146	4.5	103
+67 to 105	18.2	45	3.4	14.8	184	212	33				
+105 to 149	17.1	42	2.5	14.6	189	159	31				
+149 to 209	18.2	42	2.7	15.5	212	175	25				
+209 to 231	17.0	43	2.6	14.4	192	169	26				
+231 to 252	17.2	43	2.7	14.5	211	208	24	275		3.9	108
+252 to 280	17.1	44	2.7	14.4	212	187	23				
+280 to 317	17.1	43	2.7	14.4	204	186	25				
+317 to 340	17.3	40	2.6	14.7	223	201	29				
+340 to +360	14.8	45	2.5	12.3	170	154	34	270	140	4.0	109
	Infusion completed.										

* Subject O.G., 71 kg white male on normal NaCl (approx. 6.0 g/day) intake.

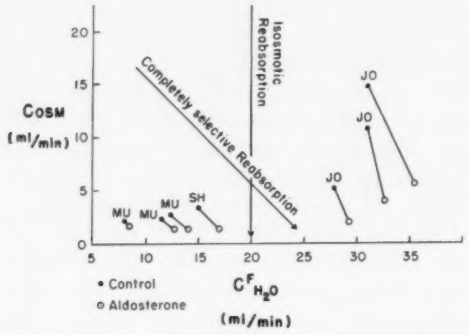


FIG. 3. EFFECT OF INTRAVENOUS ALDOSTERONE ON THE RELATION OF OSMOLAR TO FREE WATER CLEARANCE DURING A MAINTAINED WATER DIURESIS. Plotted from control periods taken at the height of water diuresis and from an average of two or three experimental periods at the peak of aldosterone effect. After aldosterone the decrement in osmolar clearance approaches or is equal to the increment in free water clearance in most of the studies. The rise in free water clearance occurs in the face of a falling osmolar clearance. The findings suggest that aldosterone abstracts sodium chloride from the urine without water.

and persisted during the periods of maximal sodium retention in only four of the seven experiments (Tables I and II; Figures 1 and 2).

The magnitude of the increased potassium excretion produced by aldosterone ($\Delta U_K V$) was small, ranging from + 12 to + 40 μEq per minute or from + 17 to + 30 per cent of the control values. The increments in K^+ excretion were of similar degree in all studies and were of considerably lesser magnitude than the decrements observed in sodium chloride excretion.

The urinary pH was observed to fall following aldosterone excretion (Tables I and II). The average observed fall was 0.38 pH units at the peak of the action of the hormone on total solute excretion.

Effect on urine flow, total solute clearance and free water excretion. In all studies aldosterone was administered only after the peak of water diuresis had been achieved. The control rate of urine flow varied considerably in different subjects from 10 to 45 ml per minute. These individual variations in urine flow seemed to be related to the variations in filtered load of solute (24). After aldosterone administration, the urine flow either did not change or showed a slight decrease, such as that which occurs in prolonged water diuresis. Therefore, the effects of aldosterone were observed during the descending limb of water diuresis.

The changes in total solute excretion after aldosterone paralleled the changes in sodium excretion both in magnitude and in phase. Thus, after an initial delay of from 20 to 60 minutes a progressive and marked reduction in urinary osmolarity ensued reaching a nadir 2 to 4 hours after the aldosterone administration. Urinary osmolarity fell to levels well below that which would be expected from water diuresis alone (21–23) (20 μOsm per ml). Thus, aldosterone facilitated the formation of an extremely dilute urine with an increased urine/plasma osmolar gradient. The urine/plasma sodium concentration gradient increased markedly in like manner.

As was the case with sodium chloride excretion, the total solute excretion did not fall appreciably in the sodium-depleted subject. However, in the other six studies total solute concentration was reduced by from 49 to 67 per cent of control values. A similar reduction in the total solute clearance (C_{osm}) was thus observed.

Since the urine flow did not change appreciably while the osmolar clearance decreased markedly, an increase in free water clearance was produced by aldosterone. In the first six experiments, the free water clearance was increased by from 1.1 to 4.3 ml per minute, but in the sodium-depleted subject, M.U., the observed changes were again slight. Since the urine flow was not rising, the calculated increases in free water cannot be attributed to an increasing water diuresis, and because solute excretion was falling, they cannot be the result of an increased solute load (24). Hence, these results imply the hypertonic abstraction of solute from the urine, i.e., reabsorption of solute unaccompanied by isosmotic amounts of water.

In Figure 3 the relationship between the osmolar clearance and the free water clearance before and after aldosterone for all seven experiments is plotted. It can be seen that after aldosterone the fall in solute clearance (ΔC_{osm}) is equal to, or almost equal to, the rise in free water clearance (ΔC_{H_2O}). In fact, in four studies of Subjects M.U. and S.H., 1 ml of free water was added to the urine for each milliliter of urine cleared of solute. This implies that aldosterone

caused the virtually totally selective abstraction of solute without water from the tubular urine.

Relationship of changes in sodium and chloride clearances to changes in osmolar clearance. Because in normal individuals sodium and chloride are the major osmolar constituents of urine and plasma and comprise the bulk of the filtered load, these solutes are the only ones available for tubular reabsorption in amounts sufficient to account for most of the free water generated during water diuresis (13). Therefore, one might assume that the changes in osmolar and free water clearance produced by aldosterone were due to the effects of the hormone on sodium (and chloride); and, in fact, in all experiments, changes in $U_{Na}V$ and in $U_{Cl}V$ closely paralleled changes in $U_{osm}V$. Aldosterone caused alterations in sodium and chloride excretion of such magnitude that, for the most part, alterations in C_{osm} produced by the hormone can be interpreted largely in terms of these ions.

In an effort to relate graphically the effect of aldosterone on sodium excretion to that on solute excretion (Figure 4), twice the C_{Na} [2] caused by the hormone has been plotted against the C_{osm} caused by the aldosterone for all experiments, with a resultant linear relationship. It can be seen that the changes of C_{osm} and thus of free water can be interpreted to be mainly a result of the effect of aldosterone on sodium (with equivalent anions). Hence, it can be concluded that aldosterone produced a fall in C_{osm} and a rise in C_{H_2O} by the abstraction of salt from a distal portion of the nephron.

Effect on urea excretion. In all experiments, the urea clearance declined gradually with no acute change observed following aldosterone administration. These data are not given in the tables.

DISCUSSION

A number of the observations made in the present study appear to confirm and extend the findings of other investigators. Thus, the delayed onset of action (3, 7, 25–29), the lack of effect on renal hemodynamics (2, 28, 30), the sodium and chloride retention, and the kaliuresis of aldosterone (1–13) have all been described under a variety of experimental and clinical conditions.

In the present study a latent period of about 20 to 60 minutes was observed before there was any demonstrable effect of the hormone on sodium excretion. The peak effect occurred in 2 to 4 hours, and the action lasted for as long as 6 to 8 hours after administration. In previous studies of man, other investigators have also noted a similar latent period and a similar duration of activity (3, 7, 25). Moreover, Barger, Berlin and Tulenko (26) and Ganong, Mulrow and Hollinger (31, 32), utilizing renal artery infusions, and Crabbé (27), utilizing an isolated toad bladder, have also noted the delayed onset of action of the hormone.

Mineralocorticoids such as desoxycorticosterone and aldosterone, in short-term studies, do not appear to alter renal hemodynamic function either in experimental animals (28–33) or in man (34–36). In the present study aldosterone did not produce significant changes in the renal plasma flow or in the glomerular filtration rate. There-

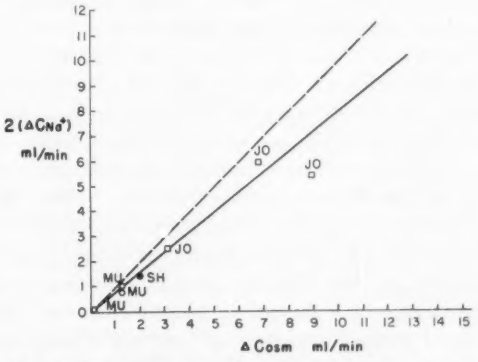


FIG. 4. RELATIONSHIP BETWEEN THE CHANGE IN OSMOLAR CLEARANCE AND TWO TIMES THE CHANGE IN SODIUM CLEARANCE PRODUCED BY ALDOSTERONE. Two times the decrement in sodium clearance after aldosterone administration is plotted against the corresponding decrement in osmolar clearance produced by the hormone. The plot illustrates that the observed changes in osmolar clearance produced by the hormone are largely accounted for by a similar change in sodium reabsorption with attendant anion.

[2] For a ΔC_{osm} of 1 ml, $1 \times P_{osm}$ (approximately 280 μOsm) was abstracted from tubular lumen. For a ΔC_{Na} of 1 ml then, $1 \times P_{Na}$ (approximately 140 μEq) of Na^+ was abstracted. Each microequivalent is an osmotically active particle. In abstraction of Na^+ an equivalent number of anions, also osmotically active particles, was removed. Hence, to relate ΔC_{Na} to ΔC_{osm}, one must multiply $\Delta C_{Na} \times 2$.

fore, it would appear that the observed effects on electrolyte and water excretion are the result of induced alterations in the activity of tubular transport systems.

The intravenous administration of aldosterone caused increased tubular reabsorption of sodium chloride and a kaliuresis. The increased potassium excretion often began prior to salt retention, was relatively small in degree, and at times had ceased before the sodium retention had reached its peak. Similar effects of aldosterone have been described both during acute administration (*2*, *7*, *25*, *26*, 31–33) and in more prolonged balance studies (*1*, 3–6, *37*).

In the present study aldosterone usually caused sodium retention in excess of chloride, confirming the results of August and Nelson (*7*), who administered aldosterone intravenously with isotonic saline infusions. It thus appears that aldosterone acted to augment the active renal tubular reabsorption of sodium ion with chloride. Precedent for the stimulation of active sodium transport by aldosterone has been found *in vitro* by Crabbé (*27*), using an isolated toad bladder.

According to present concepts (38), filtered potassium is completely reabsorbed before the potassium destined for excretion is secreted into the tubular urine by a distal K^+ for Na^+ ion exchange process. However, while the kaliuresis observed in these experiments may have resulted from an acceleration of this ion exchange mechanism, considerable evidence indicates that the predominant effect of aldosterone—i.e., to cause sodium and chloride reabsorption—occurred independently of, and at a different locus in the distal nephron from the K^+ for Na^+ ion exchange process. Thus, the kaliuresis in general was not temporally related to the sodium retention, the magnitude of the kaliuresis was far less than the sodium retention, and the degree of kaliuresis in each subject was not related to the corresponding degree of sodium retention. Ion exchange, Na^+ for K^+, does not explain the significant chloride retention which occurred concomitant with sodium reabsorption. Furthermore, ion exchange does not explain the reduced osmolarity of the urine after administration of the hormone, since the observed decrements in NaCl excretion were, in fact, accompanied by comparable reductions in total solute excretion.

The present study demonstrates that aldosterone can cause the abstraction of sodium chloride without water from the tubular urine, a finding which has not been reported heretofore.[3] During maintained water diuresis aldosterone caused the removal of sodium chloride from the urine, producing a fall in osmolar clearance while urine flow was maintained; this resulted in a concomitant rise in free water clearance. For Subjects S.H. and M.U., the relationships between solute abstraction and free water formation were such (Figure 3) as to suggest that salt was abstracted virtually *without* water. However, in Subject J.O. the increment in free water clearance was less than the decrement in osmolar clearance indicating that, in this subject, in addition to selective sodium chloride reabsorption, some additional isosmotic reabsorption may also have been induced.

Previous studies have indicated (24, 39, 40) that, during water diuresis, there is a direct relationship between solute excretion and free water clearance, a rise in C_{osm} resulting in a rise in C_{H_2O}. Therefore, the rise in free water clearance noted after aldosterone may have more significance because it occurred in the face of a falling solute excretion. Further, because the hormone was administered only after a peak of water diuresis was achieved, when urine flow was either stable or declining, the increment in free water excretion after aldosterone administration cannot be simply the result of an increasing water diuresis.

Because aldosterone facilitates net abstraction of sodium chloride without water from the urine, it thus appears to act in the nephron at a locus distal to the site of isosmotic reabsorption. The concept that free water is formed in the more distal tubular segments by selective reabsorption of solute without water is well supported by a number of studies (14–17). Micropuncture studies have shown that in the proximal tubule, sodium reabsorption is isosmotic (14–16), while fluid from the early distal tubule has been found to be hypotonic regardless of whether the final urine is hypo-or hyperosmotic (16). Vander and coworkers, using a stop-flow technique (41), have reported that in adrenalectomized dogs, aldosterone increased the maximal concentration gra-

[3] Dingman and associates measured the osmolar clearance during infusion of aldosterone. However, water diuresis was not maintained in these studies (*2*).

dient for sodium which could be developed between the plasma and distal tubular urine.

Because the hormone caused a distal abstraction of salt without water from the urine, an increased plasma/urine sodium concentration gradient was formed and the urine became more dilute. The results thus suggest that aldosterone is concerned with the production of a hypotonic urine. Inferential support for this role of aldosterone in urinary dilution may be derived from previous studies of electrolyte and water metabolism in adrenalectomized dogs (29, 30, 42, 43) and in patients with adrenal or pituitary insufficiency (35, 44, 45). In this group a characteristic picture is seen with low glomerular filtration, increased loss of sodium chloride (43), and a deficient ability to excrete a water load (47). Mineralocorticoid (46) alone, while promoting salt retention, does not repair the defect in water excretion. Cortisol (20, 30, 44, 46) increases the filtration rate, increases filtered sodium load, and allows more salt to be reabsorbed from the distal tubule with the production of free water and the excretion of a more dilute urine. However, this process is not very efficient, and although the ability to excrete water is improved, this occurs at the expense of substantial urinary wastage of sodium chloride. With cortisol plus a mineralocorticoid (35, 45), solute (i.e., NaCl) can be more efficiently removed from the distal tubule with both the conservation of sodium and the simultaneous production of a potentially more hypotonic urine for excretion. A similar function for the endogenous adrenal mineralocorticoid hormone, aldosterone, is implied by these data.

SUMMARY

Aldosterone (2 mg *d,l*-monoacetate) was given intravenously during a maintained water diuresis to normal subjects. No significant changes in glomerular filtration rate or in renal plasma flow were observed.

After a latent period of from 20 to 60 minutes, during which natriuresis actually occurred in two of the seven studies, the hormone produced a marked reduction in sodium with a lesser reduction in chloride excretion. The peak of this effect occurred in 2 to 4 hours.

Aldosterone also increased potassium excretion, but this effect was often temporally dissociated from the major action of the hormone, which was to cause sodium and chloride retention.

After aldosterone administration urine flow either did not change or declined slightly; the osmolar clearance was reduced, and hence the free water clearance was increased. In some of the studies, the decrement in osmolar clearance approached or was equal to the increment in free water clearance produced by the hormone.

The results suggest that, under circumstances of water diuresis, aldosterone can promote net abstraction of sodium chloride from the urine, virtually without water, in a distal portion of the renal tubule.

The action of aldosterone results in the creation of an increased plasma/urine sodium concentration gradient and the formation of a more hypotonic urine. It is therefore suggested that the hormone plays a role in urinary dilution.

REFERENCES

1. Mach, R. S., Fabre, J., Duckert, A., Borth, R., and Ducommun, P. Action clinique et métabolique de l'aldostérone (electrocortine). Schweiz. med. Wschr. 1954, **84**, 407.
2. Dingman, J. F., Finkenstaedt, J. T., Laidlaw, J. C., Renold, A. E., Jenkins, D., Merrill, J. P., and Thorn, G. W. Influence of intravenously administered adrenal steroids on sodium and water excretion in normal and Addisonian subjects. Metabolism 1958, **7**, 608.
3. Thorn, G. W., Sheppard, R. H., Morse, W. I., Reddy, W. J., Beigelman, P. M., and Renold, A. E. Comparative action of aldosterone and 9-alpha-fluorohydrocortisone in man. Ann. N. Y. Acad. Sci. 1955, **61**, 609.
4. August, J. T., Nelson, D. H., and Thorn, G. W. Response of normal subjects to large amounts of aldosterone. J. clin. Invest. 1958, **37**, 1549.
5. Desaulles, P. Comparison of the effects of aldosterone, cortexone and cortisol on adrenalectomized rats under various salt loads *in* An International Symposium on Aldosterone, A. F. Muller and C. M. O'Connor, Eds. Boston, Little, Brown, 1958, p. 29.
6. Kekwick, A., and Pawan, G. L. S. Oral aldosterone. Effect in a case of Addison's disease. Lancet 1954, **2**, 162.
7. August, J. T., and Nelson, D. H. The dual action of aldosterone on renal sodium reabsorption in normal subjects. Clin. Res. 1959, **7**, 274.
8. Conn, J. W., and Louis, L. H. Primary aldosteronism, a new clinical entity. Ann. intern Med. 1956, **44**, 1.

9. Holten, C., and Peterson, V. P. Malignant hypertension with increased secretion of aldosterone and depletion of potassium. Lancet 1956, **2**, 918.
10. VanBuchem, F. S. P., Doorenbos, H., and Elings, H. S. Primary aldosteronism due to adrenocortical hyperplasia. Lancet 1956, **2**, 335.
11. Laragh, J. H., Ulick, S., Januszewicz, V., Deming, Q. B., Kelly, W. G., and Lieberman, S. Aldosterone secretion and primary and malignant hypertension. J. clin. Invest. 1960, **39**, 1091.
12. Heinemann, H. O., Demartini, F. E., and Laragh, J. H. The effects of chlorothiazide on renal excretion of electrolytes and free water. Amer. J. Med. 1959, **26**, 853.
13. Smith, H. W. Principles of Renal Physiology. New York, Oxford Univ. Press, 1956.
14. Walker, A. M., Bott, P. A., Oliver, J., and MacDowell, M. L. Collection and analysis of fluid from single nephrons of the mammalian kidney. Amer. J. Physiol. 1941, **134**, 580.
15. Wirz, H. Location of antidiuretic action in the mammalian kidney *in* The Neurohypophysis, H. Heller, Ed. New York, Academic Press, 1957, p. 157.
16. Gottschalk, C. W., and Mylle, M. Micropuncture study of the mammalian urinary concentrating mechanism: evidence for the countercurrent hypothesis. Amer. J. Physiol. 1959, **196**, 927.
17. Wesson, L. G., Jr., and Anslow, W. P., Jr. Effect of osmotic and mercurial diuresis on simultaneous water diuresis. Amer. J. Physiol. 1952, **170**, 255.
18. Van Slyke, D. D., and Cullen, G. E. The determination of urea by the urease method. J. biol. Chem. 1916, **24**, 117.
19. Duncan, L. E., Jr., Liddle, G. W., and Bartter, F. C. The effect of changes in body sodium on extracellular fluid volume and aldosterone excretion by normal and edematous men. J. clin. Invest. 1956, **35**, 1299.
20. Johnson, B. B., Lieberman, A. H., and Mulrow, P. J. Aldosterone excretion in normal subjects depleted of sodium and potassium. J. clin. Invest. 1957, **36**, 757.
21. Bucht, H., Ek, J., Josephson, B., Thomasson, B., Varnauskas, E., and Werkö, L. Rapid infusion and renal function. Clin. Sci. 1956, **15**, 617.
22. Ladd, M. Effect of prehydration upon renal excretion of sodium in man. J. appl. Physiol. 1951, **3**, 603.
23. Rosenbaum, J. D., Nelson, W. P., III, Strauss, M. B., Davis, R. K., and Rossmeisl, E. C. Variation in the diuretic response to ingested water related to the renal excretion of solutes. J. clin. Invest. 1953, **32**, 394.
24. Kleeman, C. R., Epstein, F. H., and White, C. The effects of variations in solute excretion and glomerular filtration on water diuresis. J. clin. Invest. 1956, **35**, 749.
25. Liddle, G. W., Cornfield, J., Casper, A. G. T., and Bartter, F. C. The physiological basis for a method of assaying aldosterone in extracts of human urine. J. clin. Invest. 1955, **35**, 1410.
26. Barger, A. C., Berlin, R. D., and Tulenko, J. F. Intrarenal arterial infusion of aldosterone and antidiuretic hormone in the normal and adrenalectomized dog: Electrolyte and water excretion. Fed. Proc. 1957, **16**, 6.
27. Crabbé, J. Stimulation of active sodium transport across the isolated toad bladder by aldosterone *in vitro*. Clin. Res. 1960, **8**, 227.
28. Green, D. M., Farah, A., and Klemperer, W. W. Mechanisms of desoxycorticosterone action: Renal effects of the water-soluble glycoside. Endocrinology 1950, **47**, 281.
29. Roberts, K. E., and Pitts, R. F. The influence of cortisone on renal function and electrolyte excretion in the adrenalectomized dog. Endocrinology 1952, **50**, 51.
30. Garrod, O., Davies, S. A., and Cahill, G., Jr. The action of cortisone and desoxycortisone acetate on glomerular filtration rate and sodium and water exchange in the adrenalectomized dog. J. clin. Invest. 1955, **34**, 761.
31. Ganong, W. F., Mulrow, P. J., and Hollinger, G. W. Sodium and potassium excretion after injection of aldosterone into renal artery. Fed. Proc. 1957, **16**, 44.
32. Ganong, W. F., and Mulrow, P. J. Rate of change in sodium and potassium excretion after injection of aldosterone into the aorta and renal artery of the dog. Amer. J. Physiol. 1958, **195**, 337.
33. Barger, A. C., Muldowney, F. P., and Liebowitz, M. R. Role of the kidney in the pathogenesis of congestive heart failure. Circulation 1959, **20**, 273.
34. Huffman, E. R., Wilson, G. M., Jr., Clark, G. M., and Smyth, C. J. The acute renal effects of 9-alpha fluorohydrocortisone in human subjects with intact adrenals. J. Lab. clin. Med. 1956, **47**, 747.
35. Doorenbos, H., Streeten, D. H. P., and Conn, J. W. A water diuretic effect of mineralocorticoids in hydrocortisone-treated adrenal insufficiency. Clin. Res. 1958, **6**, 255.
36. Barnett, H. L., and McNamara, H. Electrolyte balances in a male infant with adrenocortical insufficiency and virilism. The effect of desoxycorticosterone acetate and salt therapy with special reference to potassium. J. clin. Invest. 1949, **28**, 1498.
37. Prunty, F. T. G., McSwiney, R. R., Mills, I. H., and Smith, M. A. The effects of aldosterone in Addison's disease and adrenal pseudo hermaphroditism. Lancet 1954, **2**, 620.
38. Berliner, R. W. Ion exchange mechanisms in the nephron. Circulation 1960, **21**, 892.
39. Orloff, J., Wagner, H. N., Jr., and Davidson, D. G. The effect of variations in solute excretion and

vasopressin dosage on the excretion of water in the dog. J. clin. Invest. 1958, **37**, 458.

40. De Wardener, H. E., and del Greco, F. The influence of solute excretion rate on the production of a hypotonic urine in man. Clin. Sci. 1955, **14**, 715.
41. Vander, A. J., Malvin, R. L., Wilde, W. S., Lapides, J., Sullivan, L. P., and McMurray, V. M. Effects of adrenalectomy and aldosterone on proximal and distal tubular sodium reabsorption. Proc. Soc. exp. Biol. (N. Y.) 1958, **99**, 323.
42. Loeb, R. F., Atchley, D. W., Benedict, E. M., and Leland, J. Electrolyte balance studies in adrenalectomized dogs with particular reference to the excretion of sodium. J. exp. Med. 1933, **57**, 775.
43. Roemmelt, J. C., Sartorius, O. W., and Pitts, R. F. Excretion and reabsorption of sodium and water in the adrenalectomized dog. Amer. J. Physiol. 1949, **159**, 124.
44. Kleeman, C. R., Maxwell, M. H., and Rockney, R. E. Mechanisms of impaired water excretion in adrenal and pituitary insufficiency. I. The role of altered glomerular filtration rate and solute excretion. J. clin. Invest. 1958, **37**, 1799.
45. Knopf, R., Streeten, D. H. P., and Conn, J. W. Mechanism of the water-retaining effect of mineralocorticoids. Clin. Res. 1960, **8**, 230.
46. Raisz, L. G., McNeely, W. F., Saxon, L., and Rosenbaum, J. D. The effects of cortisone and hydrocortisone on water diuresis and renal function in man. J. clin. Invest. 1957, **36**, 767.
47. Slessor, A. Studies concerning the mechanism of water retention in Addison's disease and in hypopituitarism. J. clin. Endocr. 1951, **11**, 700.

SPECIAL NOTICE TO SUBSCRIBERS

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 333 Cedar Street, New Haven 11, Conn., at once when you have a change of address, and do not omit the zone number if there is one.

SUBSTANCES STIMULATING GLUCOSE CATABOLISM BY THE OXIDATIVE REACTIONS OF THE PENTOSE PHOSPHATE PATHWAY IN HUMAN ERYTHROCYTES *

By ARIEH SZEINBERG † and PAUL A. MARKS

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, N. Y.)

(Submitted for publication January 9, 1961; accepted February 3, 1961)

Mature human erythrocytes possess the enzymes necessary to catabolize glucose to CO_2 via pentose phosphate pathway or to lactic acid via the Embden-Meyerhof pathway. These cells are deficient in certain enzymes of the tricarboxylic acid cycle. In the non-nucleated red cells, the oxidative reactions of pentose phosphate pathway appear to be the sole mechanism for glucose conversion to CO_2 and for the generation of TPNH (2, 3). Several investigators have suggested that a major factor in the control of the rate of glucose catabolism via the pentose phosphate pathway is the metabolic demand for TPNH (4–6). It has been demonstrated that addition of dyes capable of acting as electron acceptors from TPNH (i.e., methylene blue, phenazine methosulphate, pyocyanin) increases the rate of activity of this pathway in erythrocytes, liver slices and mammary glands (7–9). Stimulation of the pentose phosphate pathway by pyruvate in bovine corneal epithelium, by insulin in rat mammary tissue, and by ammonium salts in yeasts has been ascribed to supply of substrates requiring TPNH for their metabolism or to stimulation of enzyme systems which utilize TPNH (8, 10–12).

Among the systems requiring TPNH is that involved in the maintenance of glutathione in the reduced state (13). It has been demonstrated that human erythrocytes deficient in glucose-6-phosphate dehydrogenase (G6PD) are unable to maintain their level of reduced glutathione upon incubation with a variety of agents, such as acetylphenylhydrazine, primaquine, ascorbic acid, alpha naphthol, nitrofurantoin, cysteine and fava bean extract (14–19). This finding suggests that such substances might produce a relative insufficiency in the supply of TPNH either by inhibiting reactions in which TPNH is generated or by leading to an increase in the requirement for this coenzyme which exceeds the maximal capacity of cells deficient in G6PD. It is possible to distinguish between these mechanisms by determining whether these substances inhibit or stimulate the rate of oxidation of glucose by the reactions of the pentose phosphate pathway.

In this investigation a study has been made of the effect of the physiological substances—cysteine, ascorbic acid and pyruvate—and of agents which can cause hemolytic anemia—acetylphenylhydrazine, nitrofurantoin, α- and β-naphthol, primaquine and fava bean extract—on the rate of glucose oxidation via the pentose phosphate pathway by red cells. Observations have been made with erythrocytes from normal subjects and from persons with a genetically determined deficiency of G6PD. It has been found that these substances increase the oxidation rate of glucose carbon atom 1 to CO_2 by red cells, but not the rate of glucose conversion to lactic acid. The stimulatory effect of these substances is much greater with erythrocytes with normal levels of G6PD than with cells from persons with a marked deficiency of this enzyme. The increased rate of glucose oxidation caused by these compounds is not attributable to a direct action on G6PD. Rather, it was found that certain of these substances oxidize TPNH in the presence of diaphorase.

METHODS

Preparation of erythrocytes. Human venous blood, collected in siliconized tubes containing heparin, was mixed with an equal volume of 3 per cent dextran (Larco, grade HH, mol wt ca. 225,000) and 3 per cent glucose in 0.15 M sodium chloride, for separation of

* This work was supported in part by Grants CY 2332 and RG 7368 of the United States Public Health Service. A preliminary report of a portion of these data has appeared in abstract form (1).

† Visiting Scholar in Medicine. Present address: Government Hospital, Tel Hashomer, Israel.

erythrocytes from leukocytes and platelets by a method described in detail elsewhere (20). The erythrocytes were washed 3 times with 20 vol of cold 0.15 M sodium chloride solution and resuspended in Krebs-Ringer phosphate buffer, pH 7.4 (21), to a cell concentration of about 4×10^6 per mm^3. This erythrocyte suspension was contaminated with not more than 500 leukocytes per mm^3 and 10,000 platelets per mm^3.

Hemolysates, when employed, were prepared by lysis of 1 vol of washed packed erythrocytes with 4 vol of cold distilled water.

Isotope experiments. Two ml of the erythrocyte suspension in Krebs-Ringer phosphate buffer was placed in Warburg type flasks equipped with two side-arms. Glucose labeled in positions 1, 2 or 6 with C^{14} and nonlabeled glucose were placed in a side-arm together with additional substances as indicated in the tables. The final volume was made to 3 ml with Krebs-Ringer phosphate buffer. After 10 minutes' equilibration with an appropriate gas, the contents of the side-arm was tipped into the main compartment and incubation carried out at 37° C for 2 hours. At the end of this period of incubation, 1.0 ml 50 per cent trichloroacetic acid was introduced into the previously used side-arm through the gas vent of the stopper and 0.2 ml hyamine hydroxide and 0.2 ml toluene (21) were placed in the second side-arm. The contents of the side-arm containing the solution of acid was then tipped into the main compartment. Quantitative diffusion of released $C^{14}O_2$ into the hyamine hydroxide mixture was achieved by shaking the flasks at 37° C for 1 hour. The hyamine-toluene mixture was quantitatively transferred into bottles containing 1.0 ml of absolute ethanol and 5.0 ml of 0.3 per cent 2,5-diphenyloxazole and 0.03 per cent 2,2′-*p*-phenylene-*bis*-(5-phenyloxazole) in toluene. The assay of radioactivity was performed in a liquid scintillation counter with an efficiency of 60 per cent and a background of 30 cpm (22).

Lactic acid determinations were performed by an enzymatic procedure (23). An aliquot of the trichloroacetic acid extract from the incubation mixtures was centrifuged and the supernatant fluid recovered and adjusted to pH 10.0 with 1.0 N NaOH. Assay mixtures were set up in cuvets as follows: 2 ml glycine-NaOH buffer, 0.2 M, pH 10.5; 0.2 ml DPN, 1.5×10^{-2} M; 0.2 ml semicarbazide HCl, 1.0 M (adjusted to pH 10.5 with NaOH); 0.4 ml water and 0.2 ml of the test solution. The optical density at 340 mμ of each assay mixture was determined, and 0.050 ml lactic acid dehydrogenase (twice recrystallized, Nutritional Biochemicals Corp.) was added and the change in optical density at 340 mμ was followed until no further increase was observed. As standards, solutions of lithium lactate of known concentration were assayed with each set of determinations. In preliminary experiments it was established that cysteine, ascorbic acid, acetylphenylhydrazine, nitrofurantoin, α-naphthol and primaquine in the concentrations used in these studies did not interfere with the determination of lactic acid.

Glucose was estimated in barium hydroxide filtrates (24) by the O-amino-diphenyl method of Timell, Glaudemans and Currie (25). This procedure was chosen because none of the compounds studied, with the exception of acetylphenylhydrazine, interfered with the color reaction. Glucose could not be estimated in the presence of acetylphenylhydrazine by this procedure or by the anthrone method (26), by the iodometric titration method of Hagedorn-Jensen (27) or by an enzymatic procedure using "Glucostat" reagents of Worthington Biochemical Corporation (28).

G6PD and 6-phosphogluconic dehydrogenase activities were assayed spectrophotometrically (29, 30). A unit of enzyme activity is defined as the amount of enzyme required to give an increase in optical density of 0.001 per minute at 340 mμ (31). Erythrocytes from normal subjects assayed by this method had an activity of glucose-6-phosphate dehydrogenase of 10,000 to 20,000 U per g hemoglobin, while those from subjects with a fully expressed hereditary deficiency of this enzyme, 500 to 4,000 U per g hemoglobin.

Materials. Erythrocyte G6PD was purified from normal human erythrocytes by a method described in detail elsewhere (32). Spinach TPNH diaphorase was prepared according to the method of Avron and Jagendorf (33). An extract of fava bean was prepared by the method of Walker and Bowman (18). G6PD partially purified from yeast and a preparation of diaphorase prepared from *Clostridium kluyveri* were purchased from Sigma Chemical Corp. Primaquine phosphate was kindly provided to us by Winthrop Laboratories and nitrofurantoin [N(5-nitro-2-furfurylidene)-1-aminohydantoin] by Eaton Laboratories.

RESULTS

Stimulation of glucose oxidation to CO_2

Incubation of normal erythrocytes with cysteine, ascorbic acid, acetylphenylhydrazine, primaquine, nitrofurantoin, α- or β-naphthol is associated with a 3- to 18-fold increase in the rate of $C^{14}O_2$ formation from glucose-1-C^{14} (Table I). A consistently smaller increase in the rate of $C^{14}O_2$ formation is observed upon incubation of G6PD-deficient red cells under the same conditions. In the absence of added compounds, the rate of glucose oxidation to $C^{14}O_2$ is similar in normal and G6PD-deficient cells.

The increased rate of $C^{14}O_2$ formation in the presence of these compounds is not caused by an action of these agents directly on glucose. This is indicated by the observation that when glucose-1-C^{14} is incubated with these compounds without added erythrocytes or with boiled suspensions of erythrocytes no $C^{14}O_2$ is formed.

The increase in the rate of oxidation of glucose to $C^{14}O_2$ in the presence of these substances involves glucose-6-phosphate (G6P) which is re-

TABLE I

Effect of various compounds on the rate of $C^{14}O_2$ formation from glucose-1-C^{14} by erythrocytes of normal and G6PD-deficient subjects

Compound added*	Concentration	Cells from normal subjects†			Cells from mutant subjects†		
		No. of exp.	Activity of $C^{14}O_2$		No. of exp.	Activity of $C^{14}O_2$	
			Range	Mean		Range	Mean
	M		*$\mu c \times 10^{-3}/10^9$ RBC*			*$\mu c \times 10^{-3}/10^9$ RBC*	
None		9	0.28–0.75	0.41	8	0.16–0.52	0.35
Methylene blue	1.6×10^{-4}	8	7.9–25.1	16.7	6	1.8–5.2	4.0
Cysteine	3.5×10^{-2}	4	4.1–7.1	5.8	4	1.5–2.7	2.0
Ascorbic acid	2.8×10^{-2}	4	6.2–7.7	6.9	3	1.5–3.8	2.6
Acetylphenylhydrazine‡	3.3×10^{-2}	5	2.8–5.7	3.6	4	0.9–1.5	1.3
α-Naphthol‡	6.9×10^{-3}	6	3.2–5.5	4.4	4	0.9–2.4	1.7
β-Naphthol‡	6.9×10^{-3}	3	3.0–5.8	4.1	3	1.6–2.1	1.8
Primaquine phosphate‡	7.8×10^{-3}	7	0.8–2.5	1.6	4	0.43–0.90	0.64
Nitrofurantoin‡	4.2×10^{-3}	6	2.4–3.3	2.7	3	0.87–2.1	1.3

* Ascorbic acid and cysteine were added as neutralized solutions in Krebs-Ringer phosphate buffer. The other compounds were added as dry powders. Final glucose concentration, 1×10^{-2} M; amount of glucose-1-C^{14} added, 0.6 μc/flask; erythrocytes added, 6.9 to 9.0×10^9 flask; gas phase, 100% oxygen; incubation time, 2 hours.

† Normal subjects had red cell G6PD activities between 10,000 and 20,000 U/g hemoglobin. Each compound was assayed with erythrocytes of one mutant Caucasian female of Spanish extraction and a number of Negro mutants. The G6PD activity in erythrocytes of all these subjects was 1,000 to 4,000 U/g hemoglobin.

‡ These compounds are only slightly soluble in water. Their solubility in the incubation mixture used in these experiments is not known. Accordingly, the concentrations indicated represent calculations based on the amounts added and not necessarily on the amounts actually in solution.

cycled via pentose phosphate, as well as G6P formed from glucose. Addition of the various compounds to erythrocytes incubating with glucose-2-C^{14} is associated with an increased rate of $C^{14}O_2$ formation (Table II). In fact, in the presence of cysteine, ascorbic acid and acetylphenylhydrazine, the relative increase in the rate of $C^{14}O_2$ formation from glucose-2-C^{14} is higher than that from glucose-1-C^{14}. When none of these compounds is added, the ratio of the rate of formation of $C^{14}O_2$ from glucose-1-C^{14} to that from glucose-2-C^{14} is about 6, while in the presence of these compounds it decreased to about 2. A similar decrease in this ratio is observed in the presence of methylene blue. This is in agreement with the report of Brin and Yonemoto (7). The decrease in the ratio of the rate of $C^{14}O_2$ formed from glucose-1-C^{14} to that from glucose-2-C^{14} suggests that the stimulation of the recycling of pentose phosphate to G6P is greater than the stimulation of glucose carbon 1 oxidation. The present data provide no explanation of the mechanism of this effect.

Incubation of erythrocytes with glucose-6-C^{14} is associated with negligible amounts of $C^{14}O_2$ formation. Addition of methylene blue, cysteine, as-

TABLE II

Influence of various compounds on the rates of $C^{14}O_2$ formation from glucose-1-C^{14} and glucose-2-C^{14} by normal erythrocytes **

Compounds added	Activity of $C^{14}O_2$ recovered from		Ratio of $C^{14}O_2$ from glucose-1-C^{14} to $C^{14}O_2$ from glucose-2-C^{14}
	Glucose-1-C^{14}	Glucose-2-C^{14}	
	$\mu c \times 10^{-3}/10^9$ RBC		
None	0.54	0.09	6.0
Methylene blue	10.2	5.0	2.1
Cysteine	6.4	3.0	2.1
Ascorbic acid	7.0	3.5	2.0
Acetylphenylhydrazine	3.1	1.4	2.2
α-Naphthol	4.0	0.6	6.6
Primaquine phosphate	1.5	0.37	4.0
Nitrofurantoin	2.8	0.48	5.8

* Conditions of experiment are as in Table I. Glucose-1-C^{14} or glucose-2-C^{14}, 0.6 μc, was added to each flask. The results represent mean values of 3 experiments.

corbic acid, acetylphenylhydrazine, primaquine, nitrofurantoin or α- or β-naphthol to such incubation mixtures causes no detectable increase in the rate of glucose-6-C^{14} oxidation to $C^{14}O_2$. These observations are consistent with the fact that mature erythrocytes are lacking in the activity of certain enzymes of the tricarboxylic acid cycle (34). In addition, these data suggest that in mature red cells glyceraldehyde-3-phosphate which may be formed in the transketolase reaction is converted to a very limited extent, if at all, to carbon atoms 1 through 3 of G6P.

Oxygen dependence of the stimulatory effect

Stimulation of the $C^{14}O_2$ formation by the various compounds is less striking upon incubation of red cells in an atmosphere of air or nitrogen than in 100 per cent oxygen. The stimulatory effect was least marked when the cells were pretreated with carbon monoxide and then incubated in an atmosphere of 100 per cent nitrogen (Table III). The response to such treatment varied with different compounds, the stimulation by acetylphenylhydrazine is almost completely abolished, while that by nitrofurantoin is only little affected.

Effect of concentration of the stimulating compounds

The magnitude of stimulation in the rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$ is related to the concentration of the added compound. The concentration of the compounds at which maximal stimulation is observed is indicated in Table I. Increasing the concentration beyond the optimal point is associated with a decrease in the degree of stimulation. The maximal effect obtained with cysteine and ascorbic acid is observed at concentrations of these substances 500 times that in normal blood (which is 0.6 to 1.0×10^{-4} M) (35). However, a twofold increase in the rate of $C^{14}O_2$ formation is observed upon addition of these compounds in concentrations of 3×10^{-4} M.

TABLE III

Effect of treatment with carbon monoxide followed by incubation in atmosphere of nitrogen on the rate of $C^{14}O_2$ formation from glucose-1-C^{14} by normal erythrocytes *

Compound added	Activity of $C^{14}O_2$ recovered from: Control cells (gas phase, 100% O_2)	CO-treated cells (gas phase, 100% N_2)
	$\mu c \times 10^{-3}$/10^9 RBC	
None	0.51	0.11
Methylene blue	15.4	5.4
Cysteine	5.6	1.3
Ascorbic acid	7.0	1.4
Acetylphenylhydrazine	3.1	0.3
α-Naphthol	4.3	1.5
Primaquine phosphate	1.7	0.66
Nitrofurantoin	2.8	1.9

* Conditions of experiment are as in Table I. The CO-treated cells were obtained by passing carbon monoxide through the suspension of erythrocytes for 10 minutes before distribution into the flasks. The results represent mean values of 3 experiments.

TABLE IV

Effect of plasma and of pyruvate on the rate of $C^{14}O_2$ formation from glucose-1-C^{14} by normal cells *

Addition	Concentration	Activity of $C^{14}O_2$
	M	*$\mu c \times 10^{-3}$/10^9 RBC*
None		0.47
Plasma†		1.15
Sodium pyruvate	1×10^{-3}	0.95
	1×10^{-2}	1.4

* Conditions of experiment were described in Table I. The results represent mean values of 3 experiments.

† In the experiment with plasma, the Krebs-Ringer phosphate buffer was replaced by 2 ml homologous cell-free plasma. Control flasks containing plasma alone did not show any $C^{14}O_2$ formation.

The low rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$ which is observed when washed red blood cells are incubated in a Krebs-Ringer phosphate buffer might in part reflect a depletion of certain substances during the preliminary washing of the cells. Washed erythrocytes resuspended in plasma have a higher rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$ than do the same cells suspended in buffer solution (Table IV). This observation is in accord with that of Murphy (2). It is possible that this effect of plasma may be attributed to its content of substances such as ascorbic acid or cysteine, which stimulate the rate of glucose catabolism via the pentose phosphate pathway. There are, of course, other possible explanations for this effect of plasma and the problem requires further study.

Glucose utilization and lactic acid formation in presence of the stimulating compounds

The increased rate of glucose oxidation via the pentose phosphate pathway in the presence of the various compounds is not associated with an increase in glucose catabolism via the Embden-

TABLE V

Effect of various compounds on glucose utilization and lactic acid formation by normal erythrocytes *

Compound added	Lactic acid formed	Glucose utilized
	μmoles/10⁹ RBC/2 hrs	
None	1.15	0.61
Methylene blue	0.92	0.72
Ascorbic acid	0.93	0.69
Cysteine	0.97	0.59
Acetylphenylhydrazine	0.62	†
α-Naphthol	0.50	0.60
Primaquine phosphate	0.25	0.63
Nitrofurantoin	1.00	0.60

* Conditions of experiment are as in Table I. The results represent mean values of 4 experiments.
† Glucose utilization was not measured owing to lack of a method free from interference by acetylphenylhydrazine.

Meyerhof pathway. In the presence of the stimulating compounds, no comparable increase in the rate of glucose disappearance or lactic acid formation is observed (Table V). On the contrary, under these conditions, a decrease in the rate of lactic acid formation is generally found. Primaquine, acetylphenylhydrazine and α-naphthol cause a particularly significant decrease in the rate of lactic acid formation without a similar change in the rate of glucose utilization. This finding suggests that these compounds may inhibit some step in the conversion of glucose to lactic acid beyond G6P.

Stimulation of the oxidation of glucose to CO_2 by pyruvate

The addition of pyruvate to the incubation medium causes an increase in the rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$ by red cells. Several investigators working with other tissues, have reported that pyruvate may stimulate glucose catabolism via the pentose phosphate pathway (5, 10, 11). It has been suggested that this effect may result from pyruvate acting as an electron acceptor from TPNH in the presence of lactic dehydrogenase. In the present study, no evidence has been obtained that lactic acid dehydrogenase of erythrocytes can reduce pyruvate in the presence of TPNH. Lactic acid dehydrogenase activity has been assayed by the method of Wroblewski and LaDue (36). No activity of this enzyme is demonstrated in hemolysates when TPNH is used as the hydrogen donor over the pH range of 6.0 to 7.4. Similarly, no evidence has been obtained that the pyruvate stimulation of the rate of oxidation of glucose to CO_2 might be mediated by a pyridine nucleotide transhydrogenase. Thus, incubation of a hemolysate with TPNH, DPN and pyruvate does not result in a decrease of optical density at 340 mμ. This finding suggests that hemolysates are lacking a transhydrogenating enzyme which could lead to DPNH formation and permit the subsequent reduction of pyruvate under these conditions.

Stimulation of the oxidation of glucose to CO_2 by extract of Vicia fava

An increase in the rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$ is observed upon incubation of erythrocytes with an extract of fava bean (Table VI). In the main, this effect is due to a thermostable constituent of the extract, as a definite in-

TABLE VI

Effect of extract of Vicia fava on the rate of formation of $C^{14}O_2$ from glucose-1-C^{14} by normal erythrocytes *

Cells added	Unboiled extract		Boiled extract†	
	Extract added	Activity of $C^{14}O_2$	Extract added	Activity of $C^{14}O_2$
	ml	*μc × 10⁻³/vessel*	*ml*	*μc × 10⁻³/vessel*
Erythrocytes‡	0	3.1	0	3.1
Erythrocytes‡	0.25	6.9	0.25	3.1
Erythrocytes‡	0.50	8.1	0.50	6.9
Erythrocytes‡	1.0	9.0	1.0	7.2
Boiled erythrocytes§	0	0		
Boiled erythrocytes§	1.0	0.25	1.0	0
None	0.25	0.12	0.25	0
None	1.0	0.30	1.0	0

* Conditions of experiment are as in Table I.
† The extract of *Vicia fava* was heated at 100° C for 10 min prior to addition.
‡ 5 × 10⁹ Erythrocytes were added.
§ The erythrocytes were heated at 100° C for 10 min prior to addition.

TABLE VII

Influence of various compounds on the activity of G6P and 6-phosphogluconic dehydrogenases in hemolysates and of purified G6PD *

Compound added	Concentration	Activity of purified G6PD	Activity of hemolysates	
			G6PD	6-Phospho-gluconic dehydrogenase
		U/min	*U/min*	*U/min*
None		11.0	21.1	12.0
Cysteine	1×10^{-2} M	11.3	20.2	11.8
Ascorbic acid	1×10^{-2} M	10.9	21.0	12.1
Acetylphenylhydrazine	1.0×10^{-2} M	9.5	18.0	11.8
α-Naphthol	33% saturated solution†	6.5	14.0	11.9
Primaquine	1.3×10^{-3} M	9.4	19.1	11.0
Nitrofurantoin	33% saturated solution	5.4	9.7	7.3

* The assay mixture contained 0.5 ml 0.1 M $MgCl_2$, 0.1 ml of 2.5×10^{-3} M TPN, 0.5 ml of 0.01 M G6P, 0.005 ml G6PD purified from normal human erythrocytes or 0.050 ml hemolysate from a normal subject, 1.0 ml of a solution of the respective test substance in Krebs-Ringer phosphate buffer and water to the final volume of 3.0 ml. For 6-phosphogluconic dehydrogenase assay, 0.5 ml of 0.01 M 6-phosphogluconate was added instead of G6P. Saturated solutions of nitrofurantoin and α-naphthol were prepared by suspending 10 mg of the respective compounds in 10 ml of Krebs-Ringer phosphate buffer, shaking the suspensions for 10 minutes at room temperature and then centrifuging them.

† 33% Saturated solution indicates that the saturated solution of these compounds was in a threefold dilution in the final reaction mixture.

crease in the rate of $C^{14}O_2$ formation is observed in the presence of a boiled preparation. The higher rate of $C^{14}O_2$ formation upon incubation with an unboiled extract requires further elucidation, since a small quantity of $C^{14}O_2$ was also formed from glucose-1-C^{14} in the absence of erythrocytes, suggesting that the extract itself contained a system capable of oxidizing glucose to CO_2.

Studies of the mechanism of stimulation of glucose catabolism via the pentose phosphate pathway

1) Effect of the stimulatory compounds on the activity of G6PD. The possibility has been investigated that the increase in the rate of glucose oxidation to $C^{14}O_2$ caused by the various agents may be a consequence of a direct action on the activity of G6PD. No increase in the activity of preparations of this enzyme purified from erythrocytes or of the enzyme in crude hemolysates is observed upon addition of any of the compounds studied (Table VII). In fact, α-napththol and nitrofurantoin inhibit G6PD activity, a finding similar to that previously reported by Desforges, Kalaw and Gilchrist (37). Nitrofurantoin also inhibits 6-phosphogluconic dehydrogenase activity.

2) Effect of the stimulatory compounds on TPNH oxidation. The following experiments were performed to determine if the compounds stimulating glucose oxidation via the pentose phosphate pathway cause a direct or enzyme-mediated oxidation of TPNH.

a) TPNH was added to solutions of cysteine, ascorbic acid, acetylphenylhydrazine, α-naphthol, primaquine and nitrofurantoin buffered at pH 7.4 and the change of optical density at 340 mμ followed with time. Of these several compounds, only cysteine causes a non-enzymatic oxidation of TPNH. The rate of oxidation increases (but not by a linear relationship) with rising cysteine concentration to 1.0×10^{-2} M. Further increase in cysteine concentration is associated with a fall in the rate of TPNH oxidation.

b) The experiments indicated above were then performed with the addition of a preparation of a TPNH—specific diaphorase purified from spinach leaves or a diaphorase prepared from *Cl. kluyveri* which had activity with either TPNH or DPNH. The rate of oxidation of TPNH in the presence of cysteine was not influenced by the addition of either of these diaphorase preparations. In the presence of the diaphorase from spinach leaves, addition of nitrofurantoin or α-naphthol to a solution of TPNH causes a decrease in optical density at 340 mμ. This decrease is linear with time and its magnitude is related to the concentration of these compounds and to the diaphorase activity added (Figure 1). The addition of ascorbic acid, primaquine or acetylphenylhydrazine to such a mixture is not associated with a change in optical density at 340 mμ. In the presence of the dia-

phorase prepared from *Cl. kluyveri,* however, a decrease in optical density at 340 mμ is observed when ascorbic acid or primaquine, as well as nitrofurantoin or α-naphthol is added to a solution of TPNH. The change in optical density in these experiments is also linear with time and related to the concentration of the added compounds (Figure 2) and to the activity of the diaphorase. The difference in the results obtained with the two diaphorase preparations has not been elucidated. In any event, it would appear that the decrease in optical density observed in the presence of these enzymes represents oxidation of TPNH and not its decomposition by these compounds because it may be largely reversed by addition of a system capable of TPN reduction. An experiment demonstrating a reversal of the oxidation of TPNH in the presence of ascorbic acid by a subsequent addition of G6P and G6PD is illustrated in Figure 3. The plateau observed after addition of G6P and G6PD probably represents an equilibrated

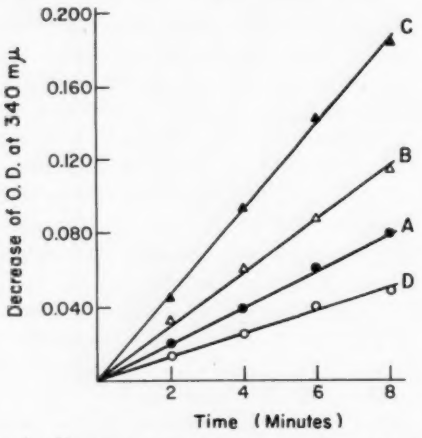


FIG. 1. EFFECT OF NITROFURANTOIN AND α-NAPHTHOL ON TPNH OXIDATION IN THE PRESENCE OF SPINACH-LEAF DIAPHORASE. The assay mixture contains Krebs-Ringer phosphate buffer, 0.15 μmole TPNH, varying volumes of a saturated solution of nitrofurantoin or α-naphthol, and diaphorase with an activity of 12 U per ml, prepared from spinach (33). The total volume is 3.0 ml and the incubation is at 24° C. Curve A, nitrofurantoin 0.100 ml, diaphorase 1.2 U; curve B, nitrofurantoin 0.25 ml, diaphorase 0.6 U; curve C, nitrofurantoin 0.25 ml, diaphorase 1.2 U; curve D, α-naphthol 1.0 ml, diaphorase 1.2 U. Control assay mixtures which contain: *1*) TPNH and diaphorase, *2*) TPNH and nitrofurantoin or α-naphthol, and *3*) diaphorase and nitrofurantoin or α-naphthol, show no change of optical density.

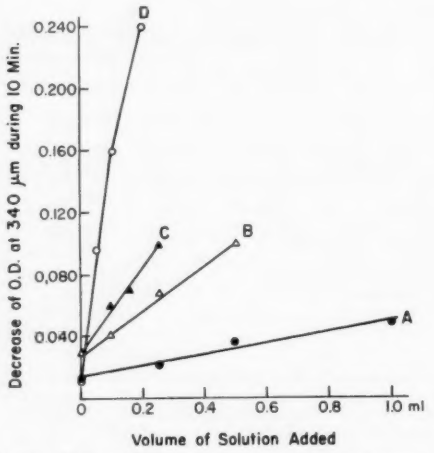


FIG. 2. EFFECT OF NITROFURANTOIN, α-NAPHTHOL, ASCORBIC ACID AND PRIMAQUINE ON TPNH OXIDATION IN THE PRESENCE OF DIAPHORASE FROM *Cl. kluyveri:* RELATIONSHIP TO VARYING CONCENTRATIONS OF THE COMPOUNDS. The assay mixture contains 0.15 μmole TPNH, diaphorase with an activity of 45 U per ml prepared from *Cl. kluyveri,* and one of the following compounds which is added as a solution in Krebs-Ringer phosphate buffer, pH 7.4: α-naphthol and nitrofurantoin, saturated solutions (prepared as indicated in footnote to Table VII); primaquine phosphate, 2×10^{-3} M; or ascorbic acid, 5.7×10^{-4} M. The volume is made up to 3 ml with the buffer solution. Curve A, α-naphthol, diaphorase 0.45 U; curve B, primaquine, diaphorase 0.9 U; curve C, nitrofurantoin, diaphorase 0.9 U; curve D, ascorbic acid, diaphorase 0.45 U. The control assay mixture from which diaphorase is omitted shows no change in optical density. Control assay mixtures containing only TPNH and diaphorase show a slight decrease in optical density which is related to the diaphorase concentration. This decrease in optical density is indicated on the ordinate at zero abscissa value.

state between the oxidation of TPNH by ascorbic acid and diaphorase and the reduction of TPN by G6P and G6PD. Addition of more G6PD after the plateau is reached results in shifting this equilibrium toward a higher TPNH concentration. Similar results are obtained with nitrofurantoin, α-naphthol and primaquine.

3) Preventing hemoglobin conversion to choleglobin. It has been reported that incubation of hemoglobin with ascorbic acid in the presence of oxygen results in its conversion to choleglobin, probably by a process of coupled oxidation and formation of hydrogen peroxide (38–41). Acetylphenylhydrazine and cysteine may also cause an oxidative breakdown of hemoglobin. Recently,

Mills and Randall have reported purifying from red cells of rat and of cow a GSH peroxidase, which catalyzes the reduction of H_2O_2 by reduced glutathione (GSH) leading to the formation of oxidized glutathione (GSSG) (40, 42, 43). Addition of GSH or GSSG plus a system generating TPNH to hemolysates of rat erythrocytes prevented the conversion of hemoglobin to choleglobin by ascorbic acid. However, in the present study this mechanism could not be demonstrated in hemolysates prepared from human erythrocytes. Addition of GSH does not decrease the rate of choleglobin formation in the presence of ascorbic acid (Figure 4). Indeed, a slight increase in the rate of choleglobin formation was observed upon addition of GSH.[1] TPNH does afford protection against choleglobin formation, but a similar activity was exerted by TPN. Thus, no specific requirement for the reduced form of TPN to prevent the conversion of hemoglobin to choleglobin could be demonstrated in hemolysates of human red cells.

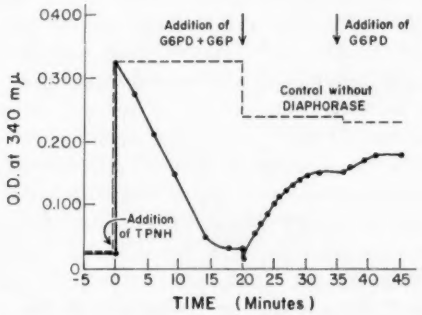


FIG. 3. REVERSAL OF THE DECREASE IN OPTICAL DENSITY AT 340 Mμ OF A MIXTURE OF TPNH, ASCORBIC ACID AND DIAPHORASE BY ADDITION OF G6P AND G6PD. The initial assay mixture (from −5 to 0 minutes) contains 12 μmoles of ascorbic acid and 0.45 U of diaphorase prepared from *Cl. kluyveri*. The control mixture contains no diaphorase (broken line). At zero time, 0.15 μmole of TPNH is added. At 20 minutes 0.5 ml of 0.1 M $MgCl_2$, 0.5 ml of 0.01 M G6P and 0.050 ml of G6PD (containing 30 U of enzyme activity) are added. This addition is associated with a decrease in optical density in both mixtures which probably reflects the dilution of the assay mixtures. At 36 minutes, an additional 60 U of G6PD is added to both test and control mixtures.

[1] Formation of oxidation products of hemoglobin upon incubation of crystalline hemoglobin with GSH has also been observed by Mills and Randall, but it was prevented by addition of purified GSH peroxidase (41).

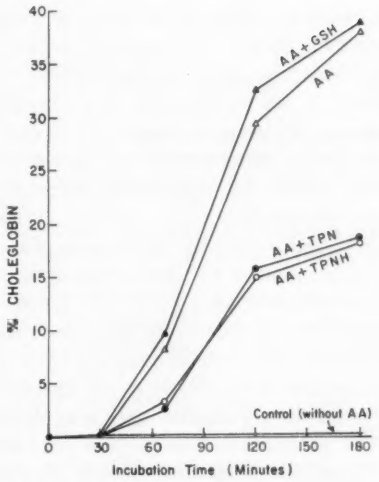


FIG. 4. THE EFFECT OF PYRIDINE NUCLEOTIDES AND OF GSH ON THE RATE OF CHOLEGLOBIN FORMATION IN THE PRESENCE OF ASCORBIC ACID. The incubation mixtures contain Krebs-Ringer phosphate solution, hemolysates prepared from normal human erythrocytes, and additions as indicated adjacent to each curve. The final concentrations of the reagents are: hemoglobin, 1.0 g per 100 ml; ascorbic acid (AA), 2.8×10^{-3} M; TPN and TPNH, 3.0×10^{-4} M; and GSH, 1.0×10^{-3} M. The final volume of the solution is 3 ml. The incubation is at 37° C. A similar pattern of results, i.e., a decrease in the rate of choleglobin formation in the presence of TPN and TPNH, but not of GSH, is obtained with two additional sets of conditions. *1*) Hemoglobin, 0.1 g per 100 ml; ascorbic acid, 7.2×10^{-4} M; TPN and TPNH, 3.0×10^{-4} M; and GSH, 8.0×10^{-4} M. *2*) Hemoglobin, 1.0 g per 100 ml; ascorbic acid, 3.6×10^{-3} M; TPN and TPNH, 3.0×10^{-4} M; and GSH, 8.0×10^{-4} M. The method of Lemberg, Legge and Lockwood (44) is used for choleglobin determination.

DISCUSSION

The present study indicates that the rate of oxidation of glucose via the pentose phosphate pathway may be selectively stimulated by cysteine, ascorbic acid or pyruvate, as well as by methylene blue, acetylphenylhydrazine, α- and β-naphthol, primaquine phosphate and nitrofurantoin. These observations suggest that among the determinants of the activity of the oxidative reactions of the pentose phosphate pathway in human erythrocytes *in vivo* may be the concentration of certain physiological substances, such as ascorbic acid and cysteine as well as a variety of agents or their metabolic products, to which the cells may become exposed.

The mode of action of the various compounds in stimulating the rate of glucose catabolism via the pentose phosphate pathway may differ. This is suggested by the observation that conversion of hemoglobin to carboxyhemoglobin followed by incubation in an atmosphere of nitrogen affects to a different extent the stimulatory activity of the various compounds.

It is probable that the increase in the rate of oxidation of glucose to CO_2 results from a more rapid rate of reoxidation of TPNH formed in the oxidative steps of the pentose phosphate pathway. It was found that cysteine may lead to a non-enzymatic oxidation of TPNH, while nitrofurantoin, ascorbic acid, primaquine and α-naphthol may cause TPNH oxidation in the presence of a suitable diaphorase. Scott and Griffith have purified a diaphorase from human erythrocytes, which catalyzes the reduction of methemoglobin by TPNH (although at a much slower rate than by DPNH) (45). The activity of nitrofurantoin, ascorbic acid, α-naphthol and primaquine in the presence of such a diaphorase from human erythrocytes has not yet been investigated. Such studies are necessary to determine the applicability of the present findings with plant or bacterial diaphorase in explaining the metabolic events in human erythrocytes. It is of interest that α-naphthol and nitrofurantoin inhibit G6PD activity yet increase the rate of oxidation of glucose-1-C^{14} to $C^{14}O_2$. This suggests that the inhibition of the activity of the enzyme by these agents is insufficient to make the enzyme activity rather than the concentration of TPN the factor limiting the rate of glucose oxidation by erythrocytes.

A number of investigators have concluded that the reoxidation of TPNH is the limiting factor in the rate of oxidation of glucose by the pentose phosphate pathway in a variety of tissues (4–12). This conclusion is based primarily on the observations that substances which may serve as electron acceptors from TPNH cause a marked increase in the rate of oxidation of glucose carbon atom 1 to CO_2. The present findings are consistent with this hypothesis. In both normal cells and cells with a deficiency of G6PD, addition of compounds which can increase the rate of TPNH reoxidation is associated with an increase in the rate of oxidation of glucose to CO_2. However, in the cells with low G6PD levels, the increment in the rate of glucose oxidation which may be achieved upon addition of the various stimulatory compounds appears to be limited by the activity of this enzyme.

There is another aspect of this stimulation of glucose catabolism via the pentose phosphate pathway which is of possible significance. A selective decrease in the activity of G6PD occurs as red cells age *in vivo* (46). Old erythrocytes, as well as cells with a genetically determined deficiency in G6PD (47) have an increased susceptibility to destruction under normal conditions and, particularly, upon exposure to a variety of agents including cysteine, acetylphenylhydrazine, nitrofurantoin, α- and β-naphthol, primaquine and the fava bean. This has suggested that the dehydrogenase reactions of the pentose phosphate pathway play an important role in the maintenance of the integrity of the red cell (14, 46, 47). It is generally presumed that this is so because these reactions provide TPNH whose reductive potential, either directly or possibly through the generation of reduced glutathione, provides protection against the damaging effects of oxidative agents (14, 17, 40–43, 48–54). The present study suggests that one mechanism by which these substances could lead to a decreased survival time of red cells is by reoxidizing TPNH. In the cells with low levels of G6PD the capacity to generate TPNH may be inadequate to provide the necessary reductive potential to protect against the oxidative effects of these compounds. The finding that TPN or TPNH, but not GSH, could protect hemoglobin against denaturation to choleglobin in the presence of ascorbic acid suggests that the protection afforded by pyridine nucleotides need not be mediated through GSH. It has been demonstrated that TPN and TPNH, but not GSH, are important to the stability of proteins other than hemoglobin, e.g., G6PD (32, 55, 56).

SUMMARY

The rate of oxidation of glucose-1-C^{14} and of glucose-2-C^{14} to $C^{14}O^2$ by erythrocytes is stimulated by certain physiological substances such as cysteine, ascorbic acid and pyruvate, as well as by agents which can cause acute hemolytic anemias, namely, primaquine, nitrofurantoin, acetylphenylhydrazine, α- and β-naphthol and an extract of fava bean.

This stimulation is more marked with cells with normal levels of glucose-6-phosphate dehydrogenase (G6PD) than with cells deficient in this enzyme. These compounds cause no increase in the rate of glucose utilization or in the rate of lactic acid formation.

The increased rate of catabolism of glucose via the pentose phosphate pathway caused by these agents is not a result of a direct effect on G6PD activity. Indeed, α-naphthol and nitrofurantoin inhibit G6PD activity. Evidence has been obtained to suggest that the stimulation of the pathway is caused by an increased rate of reoxidation of TPNH in the presence of these compounds. A diaphorase-mediated oxidation of TPNH by nitrofurantoin, α-naphthol, ascorbic acid and primaquine has been demonstrated. Cysteine can reoxidize TPNH even in the absence of a diaphorase. The increased rate of reoxidation of TPNH may be a factor in the mechanism by which these compounds lead to the destruction of aged red cells and erythrocytes with a genetically determined deficiency in G6PD.

ACKNOWLEDGMENT

The skillful help of Miss Theresa Fiorino is gratefully acknowledged.

REFERENCES

1. Szeinberg, A., and Marks, P. A. Study of the role of the pentose phosphate pathway in red cell metabolism. Clin. Res. 1960, **8**, 248.
2. Murphy, J. R. Erythrocyte metabolism. II. Glucose metabolism and pathways. J. Lab clin. Med. 1960, **55**, 286.
3. Johnson, A. B., and Marks, P. A. Glucose metabolism and oxygen consumption in normal and glucose-6-phosphate dehydrogenase deficient human erythrocytes. Clin. Res. 1958, **6**, 187.
4. Dickens, F. Part II. Enzymes in red blood cells. Recent advances in knowledge of the hexose monophosphate shunt. Ann. N. Y. Acad. Sci. 1958, **75**, 71.
5. Holzer, H. Carbohydrate metabolism. Ann. Rev. Biochem. 1959, **28**, 171.
6. deDuve, C., and Hers, H. G. Carbohydrate metabolism. Ann. Rev. Biochem. 1957, **26**, 149.
7. Brin, M., and Yonemoto, R. H. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. J. biol. Chem. 1958, **230**, 307.
8. McLean, P. Carbohydrate metabolism of mammary tissue. III. Factors in the regulation of pathways of glucose catabolism in the mammary gland of the rat. Biochim. biophys. Acta 1960, **37**, 296.
9. Cahill, G. F., Jr., Hastings, A. B., Ashmore, J., and Zottu, S. Studies on carbohydrate metabolism in rat liver slices. X. Factors in the regulation of pathways of glucose metabolism. J. biol. Chem. 1958, **230**, 125.
10. Kinoshita, J. H. The stimulation of the phosphogluconate oxidation pathway by pyruvate in bovine corneal epithelium. J. biol. Chem. 1957, **228**, 247.
11. Wenner, C. E. Oxidation of reduced triphosphopyridine nucleotide by ascites tumor cells. J. biol. Chem. 1959, **234**, 2472.
12. Holzer, H., and Witt, I. Beschleunigung des oxydativen Pentosephosphatcyclus in Hefezellen durch Ammoniumsalze. Biochim. biophys. Acta 1960, **38**, 163.
13. Rall, T. W., and Lehninger, A. L. Glutathione reductase of animal tissues. J. biol. Chem. 1952, **194**, 119.
14. Beutler, E. The hemolytic effect of primaquine and related compounds: A review. Blood 1959, **14**, 103.
15. Zinkham, W. H., and Childs, B. A defect of glutathione metabolism in erythrocytes from patients with a naphthalene-induced hemolytic anemia. Pediatrics 1958, **22**, 461.
16. Kimbro, E. L., Jr., Sachs, M. V., and Torbert, J. V. Mechanism of the hemolytic anemia induced by nitrofurantoin (Furadantin). Further observations on the incidence and significance of "primaquine-sensitive" red cells. Bull. Johns Hopk. Hosp. 1957, **101**, 245.
17. Szeinberg, A., Adam, A., Ramot, B., Sheba, C., and Myers, F. The incorporation of isotopically labelled glycine into glutathione of erythrocytes with glucose-6-phosphate dehydrogenase deficiency. Biochim. biophys. Acta 1959, **36**, 65.
18. Walker, D. B., and Bowman, J. E. *In vitro* effect of *Vicia faba* extracts upon reduced glutathione of erythrocytes. Proc. Soc. exp. Biol. (N. Y.) 1960, **103**, 476.
19. Mela, C., and Perona, G. P. Sulla resistenza *in vitro* del glutatione ridotto eritrocitario al succo di fave fresche; nota preventiva. Boll. Soc. ital. Biol. sper. 1959, **35**, 146.
20. Marks, P. A., Gellhorn, A., and Kidson, C. Lipid synthesis in human leukocytes, platelets, and erythrocytes. J. biol. Chem. 1960, **235**, 2579.
21. Cohen, P. P. *in* Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Related Methods for Study of Tissue Metabolism. Minneapolis, Burgess Publishing, 1957, p. 149.
22. Passmann, J. M., Radin, N. S., and Cooper, J. A. D. Liquid scintillation technique for measuring carbon-14-dioxide activity. Analyt. Chem. 1956, **28**, 484.
23. Horn, H. D., and Bruns, F. H. Quantitative Bestimmung von L(+)− Milchsäure mit Milchsäuredehydrogenase. Biochim. biophys. Acta 1956, **21**, 378.

24. Somogyi, M. A method for the preparation of blood filtrates for the determination of sugar. J. biol. Chem. 1930, **86**, 655.
25. Timell, T. E., Glaudemans, C. P. J., and Currie, A. L. Spectrophotometric method for determination of sugars. Analyt. Chem. 1956, **28**, 1916.
26. Scott, T. A., Jr., and Melvin, E. H. Determination of dextran with anthrone. Analyt. Chem. 1953, **25**, 1656.
27. Rappaport, F. Rapid Microchemical Methods for Blood and C.S.F. Examinations. New York, Grune and Stratton, 1942, p 242.
28. Saifer, A., and Gerstenfeld, S. The photometric microdetermination of blood glucose with glucose oxidase. J. Lab. clin. Med. 1958, **51**, 448.
29. Kornberg, A., and Horecker, B. L. Glucose-6- phosphate dehydrogenase *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1955, vol. 1, p. 323.
30. Horecker, B. L., and Smyrniotis, P. Z. Phosphogluconic acid dehydrogenase from yeast. J. biol. Chem. 1951, **193**, 371.
31. Marks, P. A., and Gross, R. T. Erythrocyte glucose-6-phosphate dehydrogenase deficiency: Evidence of differences between Negroes and Caucasians with respect to this genetically determined trait. J. clin. Invest. 1959, **38**, 2253.
32. Marks, P. A., Szeinberg, A., and Banks, J. Erythrocyte glucose-6-phosphate dehydrogenase of normal and mutant human subjects. J. biol. Chem. 1961, **236**, 10.
33. Avron, M., and Jagendorf, A. T. A TPNH diaphorase from chloroplasts. Arch. Biochem. 1956, **65**, 475.
34. Rubinstein, D., Ottolenghi, P., and Denstedt, O. F. The metabolism of the erythrocyte. XIII. Enzyme activity in the reticulocyte. Canad. J. Biochem. 1956, **34**, 222.
35. Stein, W. H., and Moore, S. The free amino acids of human blood plasma. J. biol. Chem. 1954, **211**, 915.
36. Wroblewski, F., and LaDue, J. S. Lactic dehydrogenase activity in blood. Proc. Soc. exp. Biol. (N. Y.) 1955, **90**, 210.
37. Desforges, J. F., Kalaw, E., and Gilchrist, P. Inhibition of glucose-6-phosphate dehydrogenase by hemolysis inducing drugs. J. Lab. clin. Med. 1960, **55**, 757.
38. Lemberg, R., and Legge, J. W. Hematin Compounds and Bile Pigments. New York, Interscience Publishers, 1949, p. 476.
39. Kaziro, K., Kikuchi, G., and Kawai, M. Reaction of verdohemoglobin formation *in vitro*, with special reference to its quantitative treatment. J. Biochem. (Tokyo) 1956, **43**, 161.
40. Mills, G. C. Hemoglobin catabolism: I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. biol. Chem. 1957, **229**, 189.
41. Mills, G. C., and Randall, H. P. Hemoglobin catabolism: The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. J. biol. Chem. 1958, **232**, 589.
42. Mills, G. C. The purification and properties of glutathione peroxidase of erythrocytes. J. biol. Chem. 1959, **234**, 502.
43. Mills, G. C. Glutathione peroxidase and the destruction of hydrogen peroxide in animal tissues. Arch. Biochem. 1960, **86**, 1.
44. Lemberg, R., Legge, J. W., and Lockwood, W. H. Coupled oxidation of ascorbic acid and haemoglobin. 3. Quantitative studies on choleglobin formation. Estimation of haemoglobin and ascorbic acid oxidation. Biochem. J. 1941, **35**, 339.
45 Scottt, E. M., and Griffith, I. V. The enzymic defect of hereditary methemoglobinemia: Diaphorase. Biochim. biophys. Acta 1959, **34**, 584.
46. Marks, P. A., Johnson, A. B., and Hirschberg, E. Effect of age on the enzyme activity in erythrocytes. Proc. nat. Acad. Sci. (Wash.) 1958, **44**, 529.
47. Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S. Enzymatic deficiency in primaquine-sensitive erythrocytes. Science 1956, **124**, 484.
48. Keilin, D., and Hartree, E. F. Spectroscopic study of permeability and lysis of red blood corpuscles. Nature (Lond.) 1946, **157**, 210.
49. Fegler, G. Relationship between reduced glutathione content and spontaneous haemolysis in shed blood. Nature (Lond.) 1952, **170**, 624.
50. Benesch, R. E., and Benesch, R. Relation between erythrocyte integrity and sulfhydryl groups. Arch. Biochem. 1954, **48**, 38.
51. Beutler, E., Robson, M., and Buttenwieser, E. The mechanism of glutathione destruction and protection in drug-sensitive and nonsensitive erythrocytes. *In vitro* studies. J. clin. Invest. 1957, **36**, 617.
52. Horecker, B. L., and Hiatt, H. H. Pathways of carbohydrate metabolism in normal and neoplastic cells. New Engl. J. Med. 1958, **258**, 177 and 225.
53. Marks, P. A. Red cell glucose-6-phosphate and 6-phosphogluconic dehydrogenases and nucleoside phosphorylase. Science 1958, **127**, 1338.
54. Jandl, J. H., Engle, L. K., and Allen, D. W. Oxidative hemolysis and precipitation of hemoglobin. I. Heinz body anemias as an acceleration of red cell aging. J. clin. Invest. 1960, **39**, 1818.
55. Marks, P. A., Banks, J., and Gross, R. T. Glucose-6-phosphate dehydrogenase thermostability in leukocytes of Negroes and Caucasians with erythrocyte deficiency of this enzyme. Biochem. biophys. Res. Com. 1959, **1**, 199.
56. Kirkman, H. N. Characteristics of glucose-6-phosphate dehydrogenase from normal and primaquine-sensitive erythrocytes. Nature (Lond.) 1959, **184**, 1291.

THE COMPOSITION AND BIOSYNTHESIS OF LIPIDS IN HUMAN ADIPOSE TISSUES*

By ALFRED GELLHORN AND PAUL A. MARKS

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Medical Service, Francis Delafield Hospital, New York, N. Y.)

(Submitted for publication January 9, 1961; accepted February 3, 1961)

The capacity of adipose tissue in rats and mice to synthesize fatty acids and triglycerides from acetate, glucose and long chain fatty acids and to oxidize these substrates to CO_2 has been well established (1–5). In this investigation, a study has been made of lipid biosynthesis and lipid composition of human adipose tissues.

Human subjects provide a unique opportunity to compare the metabolism of normal subcutaneous adipose tissue with a benign neoplasm of fat tissue, the lipoma. Comparative studies of most tumors with their normal tissue counterpart have been difficult because of cellular nonhomogeneity of both normal tissues and tumors and the inability to obtain tumor and normal control tissue from the same subject. The lipoma, on the other hand, arises most frequently in the subcutaneous adipose tissue and morphologically is indistinguishable from normal adipose tissue. This makes it possible to examine the synthetic and catabolic activities and composition of the tumor and control tissue in the same subject under identical environmental conditions.

In the present studies, it has been found, *in vitro,* that lipomas had a significantly higher rate of acetate incorporation into the mixed lipids than the normal subcutaneous adipose tissue of the same subject. Although the rate of synthesis of individual fatty acids was greater in the lipoma than in the normal fat, the relative distribution of radioactivity among the fatty acids was the same in the two tissues. These tissues did not differ significantly with respect to the rate of oxidation of acetate to CO_2 or the rate of transfer of newly synthesized lipids into the medium.

Despite the differences in the rate of incorporation of acetate into the mixed lipids, the fatty acid composition of the normal subcutaneous fat and the lipoma did not differ qualitatively or quantitatively. In addition, there was impressive constancy of the fatty acid composition of the normal fat and lipoma among the subjects studied despite wide variation in the rates of lipid synthesis by these tissues from one person to the next.

MATERIALS AND METHODS

Twenty patients with subcutaneous lipomas provided the material for this study. The tumors were removed under block local anesthesia together with a sample of adjacent normal adipose tissue. All patients had eaten 3 to 5 hours prior to the operation. On removal, the tissues were kept at about 5° C until they were prepared for incubation. The fibrous tissue capsules of the lipomas were dissected away and tissue slices of the tumor and the normal subcutaneous adipose tissue were prepared with a Stadie tissue slicer (6). Usually 1 hour elapsed between the operative removal of tissue and the beginning of incubation.

Incubation was carried out in 50 ml Warburg-type flasks employing rubber serum bottle stoppers to seal the flasks. One to two μc of acetate-1-C^{14}, 0.1 ml 1 M glucose and varying amounts of nonradioactive sodium acetate were placed in the side-arm. Into the main compartment were added 150 to 300 mg of tissue and an amount of Krebs-Ringer bicarbonate solution (7), containing 5 to 10 per cent of the patient's own serum, sufficient to make a final volume of 5 ml. In most experiments a flask was set up, identical with those just described except that boiled rather than fresh tissue was employed. The flasks were gassed with 95 per cent O_2, 5 per cent CO_2 for 5 minutes. After 20 minutes of temperature equilibration at 37° C in a Dubnoff metabolic shaker-bath, the reaction was begun by tipping into the main compartment the contents of the side-arm. The duration of incubation was usually 2 and 4 hours. In preliminary experiments it was established that, under the present experimental conditions, the rate of incorporation of acetate-1-C^{14} into the mixed lipids or into $C^{14}O_2$ by normal adipose tissue and lipoma slices was linear over a 6 hour period.

At the completion of incubation, the flasks were chilled in chipped ice. A 30 per cent solution of KOH was in-

* This work was supported in part by Grant CY-2332 of the National Cancer Institute, U. S. Public Health Service, The Alma Toorock Memorial Fund and the Evelyn R. Hill Fund. A portion of these data has been presented in the Proceedings of the International Symposium on Drugs Affecting Lipid Metabolism, Milan, 1960.

troduced into the center well, 1 ml 0.2 N H_2SO_4 was added to the medium in the main compartment and the flasks were shaken in ice for another 30 minutes. The KOH and H_2SO_4 additions were made by needle puncture through the rubber stopper.

In nine experiments, the radioactivity of the lipids in the tissue and in the medium was analyzed separately. In these studies, at the completion of the incubation period, the flask contents were poured through a fine-mesh, stainless steel screen. The tissue was repeatedly washed with either distilled water or dilute ammoniacal ethanol. Lipids were then extracted from the medium and tissue as described below.

$C^{14}O_2$ assay. The 0.2 ml of 30 per cent KOH solution in the center well was transferred quantitatively to centrifuge tubes and saturated $BaCl_2$ was added to precipitate the carbonate. The $BaCO_3$ was washed repeatedly with water until the samples obtained from flasks with boiled tissue (blanks) showed negligible radioactive contamination (less than 0.06 per cent of the added counts). The $BaCO_3$ was suspended in a mixture of diphenyloxazole, 0.3 per cent, and thixin, 2.5 per cent, in toluene and

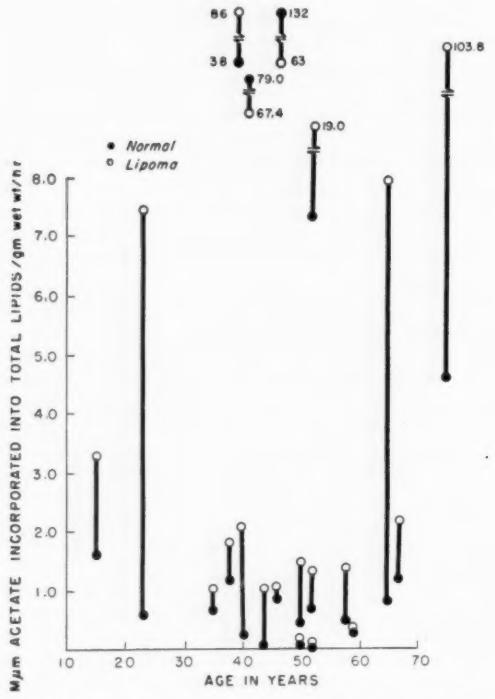


FIG. 1. THE RATE OF INCORPORATION OF ACETATE INTO NORMAL ADIPOSE TISSUE AND LIPOMA CORRELATED WITH THE AGE OF EACH OF 20 SUBJECTS. The calculation of acetate incorporated into the total mixed lipids is based on the initial specific activities of the acetate in the incubation medium and the total radioactivity incorporated into the lipids.

TABLE I

Nitrogen and lipid content of normal adipose tissue and lipoma

	No. of subjects*	Nitrogen	SEM†	Lipid	SEM†
		mg/g wet wt		*mg/g wet wt*	
Normal	20	5.85	±0.48	630	±9
Lipoma	20	2.78	±0.32	810	±9

* At least 4 aliquots of tissue were analyzed from each subject.

† SEM is the standard error of the mean.

counted in a liquid scintillation spectrometer (8). It was demonstrated in preliminary experiments that quenching of radioactivity did not occur when as much as 2.5 g $BaCO_3$ was added. Radioactivity was measured in this system with an absolute efficiency of 75 per cent and a background of 30 cpm.

Extraction of lipids. The mixed lipids of the tissues or medium were extracted and prepared for analysis by a modification of the methods described by Folch, Lees and Sloane Stanley (9). The sample to be extracted was homogenized in a multimixer employing 80 to 100 ml $CHCl_3$–CH_3OH (2:1). In order to free the lipid extracts of nonlipid radioactive compounds, they were placed in viscose tubing and dialyzed against running tap water in a rotatory dialyzer for 12 to 16 hours. It was demonstrated that this method completely removed radioactive materials from boiled tissue samples which were carried through all steps of the experimental procedure. It was also found that there was no detectable loss of C^{14}-labeled tripalmitin added to a blank sample after 24 hours of continuous dialysis.

After dialysis, the mixed lipid extract was taken to dryness under reduced pressure and made up to a known volume with petroleum ether (bp 30 to 60° C). An aliquot was taken for analysis of radioactivity and lipid content.

Lipid fractionation. Free fatty acid, triglyceride and triglyceride fatty acids were separated by a modification of the method of Borgström (10). An aliquot of the mixed lipid extract in petroleum ether was taken to dryness and redissolved in CCl_4. To obtain the free fatty acids, the CCl_4 was repeatedly washed with 0.5 per cent NH_4OH, 0.2 M KCl in 40 per cent CH_3OH. Carrier, nonradioactive palmitic acid was added to the CCl_4 to facilitate the free fatty acid extraction. The ammoniacal methanol solution of fatty acids was acidified with H_2SO_4 and the free fatty acids extracted with petroleum ether (bp 30 to 60° C). The petroleum ether extract of the fatty acids was washed with water until the wash was neutral and then dried over anhydrous Na_2SO_4.

The CCl_4 solution containing the triglycerides from which the free fatty acids had been extracted was divided into two parts, one for the determination of the radioactivity of the triglycerides and the other for hydrolysis (by refluxing in 5 per cent H_2SO_4 in CH_3OH for 2 hours) to recover the triglyceride fatty acids. The

TABLE II

The mean ratios of acetate incorporation into mixed lipids and acetate oxidation to CO_2 of lipoma to normal

No.*	Lipoma/normal					
	Acetate incorporated into mixed lipid (mμmoles/hr)			Acetate oxidized to CO_2 (mμmoles/hr)		
	Per g wet wt	Per mg N	Per g lipid-free wt	Per g wet wt	Per mg N	Per g lipid-free wt
20	4.77 ± 1.29†	13.34 ± 5.46	8.67 ± 3.40	1.10 ± 0.15	2.04 ± 0.44	1.80 ± 0.40
14						

* No. refers to the number of separate experiments on which the mean is based.
† Standard error of the mean.

radioactivity of the various lipid fractions was assayed in a liquid scintillation spectrometer employing toluene containing 0.3 per cent 2,5-diphenyloxazole and 0.03 per cent *p-bis* [2-(phenyloxazolyl)] benzene as a solvent-phosphor system.

Fatty acid analysis. An aliquot of the mixed lipid extract was hydrolyzed and methylated by refluxing for 2 to 3 hours with 5 per cent H_2SO_4 in dry methanol at 70° C. The methyl esters of the fatty acids thus formed were extracted with petroleum ether and the extract was taken to dryness in a nitrogen atmosphere. The fatty acid methyl esters were analyzed by gas-liquid chromatography at 185° C in the Barber-Coleman apparatus with a radium source ionization detector. The mobile phase was argon gas and the stationary phase was either ethylene glycol adipate or ethylene glycol succinate, 12 to 17 per cent, on 80 to 100 mesh Chromosorb W (11–13).

The determination of the radioactivity present in the individual fatty acids and their specific activities was achieved by splitting the effluent gas stream coming from the column. One part of the effluent gas passed through the detector cell, the other to an anthracene-filled glass cartridge outside of the apparatus which effectively trapped the fatty acid (14). The cartridges were changed manually to collect the desired fractions as indicated by the tracing which was being recorded simultaneously. The radioactivity on the anthracene was determined in the scintillation spectrometer by inserting the glass cartridge into the well of a Lucite sphere which had the same dimensions as the conventional liquid counting bottle.

RESULTS

Lipid biosynthesis and acetate oxidation in normal adipose tissue and lipoma. In 18 of 20 subjects, the rate of acetate incorporation into mixed lipids of lipoma was higher than that of the adjacent normal adipose tissue (Figure 1). In some persons the normal adipose tissue synthesized fat more rapidly than did the lipoma of other patients. Among the subjects studied, there was great variation in the rate of acetate incorporation into mixed lipids by normal subcutaneous adipose tissue. In the rat, it has been found that there is a decrease in the *in vitro* rate of acetate incorporation into mixed lipids by adipose tissues with aging.[1] As can be seen in Figure 1, the variation in lipid synthesis among the subjects studied bears no systematic relationship to their ages.

The rates of acetate incorporation into mixed lipids mentioned above have been related to the unit of wet weight of tissue. Normal adipose tissue was found to have a relatively higher nitrogen and lower lipid content than lipoma (Table I). When the rates of acetate incorporation into the mixed lipids are referred to these units, the difference in the rates of lipid synthesis between the two tissues are magnified (Table II). The ratio of the rate of oxidation of acetate to CO_2 by lipomas to that of the normal adipose tissue of the same subject did not differ significantly from 1.0 whether related to units of wet weight, nitrogen, or lipid-

[1] Benjamin, W., Kundel, H., and Gellhorn, A. Unpublished observations.

TABLE III

Fatty acids in normal adipose tissue and lipoma *

Fatty acids†	Normal	Lipoma	Fatty acids†	Normal	Lipoma
C12	0.35 ± 0.025	0.35 ± 0.017	C18	6.0 ± 0.267	5.3 ± 0.115
C14	2.40 ± .069	2.50 ± .053	C18-1	49.9 ± .325	49.9 ± .400
C16	24.60 ± .267	25.60 ± .354	C18-2	9.5 ± .143	9.6 ± .209
C16-1	5.60 ± .253	5.70 ± .014			

* These data are weighted mean values, the standard error of the mean for 4 to 14 separate samples of normal adipose tissue and lipoma from each of 9 subjects. The figures are given as percentages ± the standard error of the mean.
† Designation of fatty acids as in Figure 2.

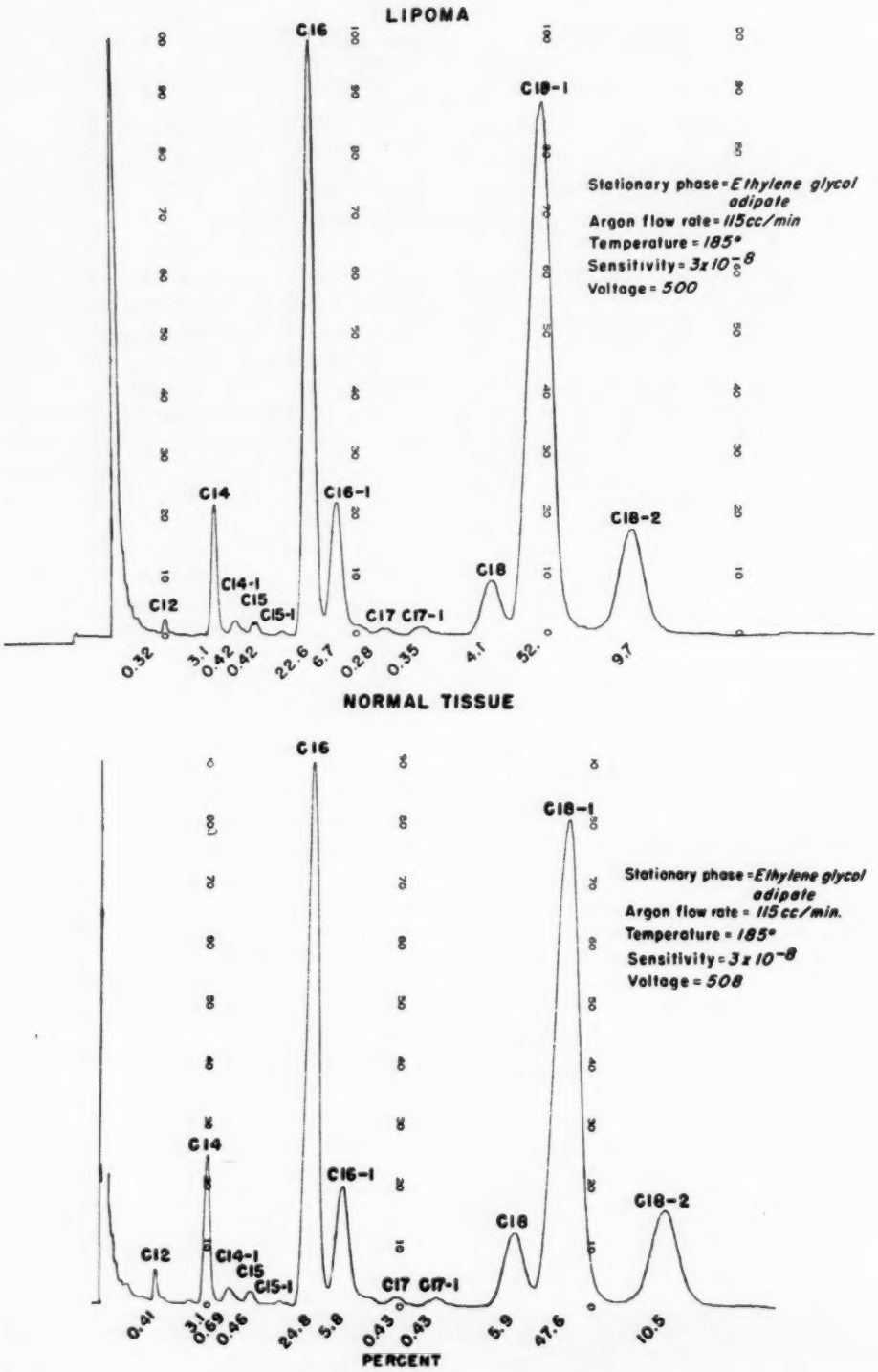


FIG. 2. See legend at bottom of page 929.

TABLE IV

Acetate-1-C^{14} incorporation into lipid components of normal adipose tissue and medium *

	Mixed lipid†	Sp. act.‡	Mixed lipid triglyceride†	Sp. act.	Mixed lipid free fatty acid†	Sp. act.	Triglyceride fatty acid†	Sp. act.
Tissue	33.0 (27.5–41.4)	0.047 (0.038–0.060)	35.0 (27.5–42.5)	0.048 (0.038–0.059)	0.35 (0.22–0.39)	0.078 (0.045–0.133)	28.4 (19.5–38.7)	0.033 (0.020–0.050)
Medium	0.35 (0.25–0.39)	0.006 (0.004–0.007)	0.050 (0.038–0.959)	0.001 (0.0009–0.0015)	0.30 (0.22–0.42)	0.026 (0.017–0.029)		

* These are data for a single experiment. The mean value for radioactivity in the total lipid and the other lipid components was calculated by multiplication of the specific activity of the component by the total milligrams present per gram wet weight of adipose tissue. The values in parentheses indicate the range of determinations on 4 separate samples.
† Values shown are $\mu c \times 10^{-3}$/g wet weight.
‡ Sp. act. = specific activity in $\mu c \times 10^{-3}$/mg.

free weights of the tissues (Table II). Thus, the increased rate of fat biosynthesis by lipomas was 5 to 13 times greater than the normal tissue, depending upon the reference unit selected; the average rate of acetate-1-C^{14} oxidation to $C^{14}O_2$ was not significantly different between lipoma and normal adipose tissue, regardless of the reference unit used for calculation.

The proportion of the newly synthesized mixed lipid radioactivity transferred to the medium by lipoma and normal adipose tissue ranged from 1 to 34 per cent and the mean ratio of lipoma: normal was 1.05. It was found that an increasing amount of the mixed lipid radioactivity appeared in the medium with time but the proportion of this, relative to the total radioactivity in the mixed lipids, remained constant.

Composition of normal subcutaneous adipose tissue and lipoma. Gas-liquid chromatographic analysis of the fatty acids from normal adipose tissue and lipoma revealed that oleic and palmitic acids predominated (Figure 2 and Table III). There were no qualitative or significant quantitative differences in the fatty acid composition of lipoma compared with normal fat. The seven fatty acids indicated in Table III accounted for about 98 per cent of all the fatty acids present in these tissues. Twenty-six fatty acids, in addition to those listed in Table III, were identified in the fat of the tissues here studied in a capillary column gas-liquid chromatographic analysis.[2] These fatty acids, however, comprise less than 2 per cent of the total fatty acid composition. No qualitative differences in these minor (by criterion of weight) fatty acid components were noted between normal adipose tissue and lipoma. The standard error of the mean shown for each fatty acid in Table III indicates the small variation noted between samples from different subjects. A statistical comparison of the variation of fatty acid composition within sources and between sources led to the conclusion that there was no greater variation in the fatty acid composition between subjects than in the same subject.

Distribution of C^{14} in lipid components of tissue and medium. More than 90 per cent of the tissue lipid radioactivity was present in the triglyceride and no more than 1.5 per cent was in the free fatty acids of the tissue (Tables IV and V). In the medium, on the other hand, the radioactivity was found predominantly in the free fatty acid fraction. Hydrolysis of the tissue triglycerides revealed that 80 per cent of the radioactivity was in the fatty acids. Although no significant difference in the distribution of the incorporated ace-

[2] We wish to acknowledge the assistance of Dr. S. R. Lipsky, Yale University School of Medicine, in this analysis and the generous advice given by him, Dr. Evan Horning and Dr. Charles C. Sweeley, National Heart Institute, Bethesda, Md., and Dr. David Turner, Sinai Hospital, Baltimore, Md., in our studies with gas-liquid chromatography.

FIG. 2. GAS-LIQUID CHROMATOGRAPHIC RECORDS OF METHYL ESTERS OF FATTY ACIDS ISOLATED FROM LIPOMA AND NORMAL ADIPOSE TISSUE. The designations above each peak identify the fatty acid according to carbon chain length (e.g., C12 dodecanoic acid) and number of unsaturated bonds (e.g., monoenoic C16-1 and dienoic C18-2). The numbers under each peak refer to the percentage of the total fatty acids represented by the particular fatty acid (e.g., C18-1, 9-octadecenoic acid or oleic acid, comprises 52 per cent of the total fatty acids in the lipoma here shown).

TABLE V

Distribution of radioactivity in lipid fractions

	Mixed lipid triglyceride/mixed lipid	Mixed lipid free fatty acid/mixed lipid	Triglyceride fatty acid/triglyceride
	%	%	%
Normal [6]*			
Tissue	99.7	0.9	79.1
Medium	20.3	78.0	
Lipoma [3]			
Tissue	90.4	1.3	109.0
Medium	11.2	78.0	

* The numbers in brackets indicate the number of experiments from which the mean values have been calculated.

tate-1-C^{14} was found between the normal tissue and the lipoma, it is apparent that in the adipose tissues of man as in the rodent (15) there is a small free fatty acid intracellular pool and a large triglyceride pool. The lipid which is transferred across the cell membrane is predominantly in the form of the free fatty acid.

All of the fatty acids isolated from adipose tissue incubated with acetate-1-C^{14} were labeled (Table VI). The rate of C^{14} incorporation into the saturated fatty acids is greater than into the unsaturated fatty acids. Although the absolute amount of radioactivity was greater in the individual fatty acids isolated from lipoma than from normal, the distribution of the radioactivity among the fatty acids as shown by the relative specific activity was essentially the same in lipoma and in normal adipose tissue. The percentage of the total radioactivity recovered in the myristic acid (C-14) of lipoma was high in one experiment and comparable to that of normal in the other. This accounts for the higher mean relative specific activity of the myristic acid of the lipoma than of the normal. The finding of small amounts of radioactivity in the essential fatty acid, linoleic (C18-2), was unexpected. This could represent contamination from other labeled fatty acids; isolation and degradation of the linoleic acid will be required for interpretation of the result.

TABLE VI

The specific activities of fatty acids of the mixed lipids of normal adipose tissue and lipoma following incubation with acetate-1-C^{14} *

Fatty acid†	Per cent of total fatty acids		Per cent of total cpm recovered		Relative sp. act.‡	
	N	L	N	L	N	L
C12	1.3	1.2	3.4	2.7	2.20	2.32
C14	3.6	3.6	9.2	23.9	2.91	7.82
C16	18.9	20.3	45.2	49.6	2.51	2.52
C16-1	13.6	10.5	7.4	3.3	0.58	0.30
C18	5.1	4.1	22.8	11.3	4.71	3.73
C18-1	47.3	43.5	3.6	5.2	0.05	0.12
C18-2	11.8	15.1	7.1	2.1	0.74	0.16

* Results of 2 experiments; all analyses were determined at least in duplicate. N = normal, L = lipoma.

† Designation of fatty acids as in Figure 2.

‡ The relative specific activity represents: $\frac{\%\text{ of total cpm recovered}}{\%\text{ of total fatty acids}}$

DISCUSSION

This study demonstrates that normal adipose tissue of man can incorporate acetate into lipids *in vitro*. The rate of acetate incorporation per gram normal adipose tissue per hour among the subjects studied ranged from 0.1 to 130 mμmoles. This may be compared to 34 and 23 mμmoles acetate incorporation per gram wet weight per hour of human leukocytes and platelets, respectively (16). Although the median *in vitro* rate of about 1 mμmole acetate incorporated per gram adipose tissue per hour does not seem high, recognition that this tissue constitutes 20 per cent of the body weight provides a better indication of its importance in fat metabolism.

In the human subjects whose subcutaneous adipose tissue was studied, there was great variation in the *in vitro* rate of acetate incorporation into the mixed lipids. The epididymal fat pads from inbred strains of rats maintained under constant laboratory conditions and on a standard diet have a less variable *in vitro* rate of fat biosynthesis when the tissue from animals of the same age are studied. With aging, this rate declines from a mean of 7 μmoles per g epididymal adipose tissue in animals weighing 80 g, to 0.7 μmoles in 300 g animals.[1] In the present studies of human adipose tissue, no correlation between fat synthesis and age, sex, or ethnic origin could be demonstrated.

In contrast to the variability of the rates of lipid synthesis from one human subject to another, was the small deviation in the fatty acid composition of the adipose tissue from one person to the next. Observations of Moore and Cook on human subjects in Scotland showed essentially the same fatty acid composition as that reported here (17); Hirsch and co-workers have also emphasized the constancy of the fatty acid pattern in human adipose tissue (18). The uniformity of the adipose tissue fatty acid composition may reflect an overall similarity of the dietary fat intake among the subjects studied. The patients were, however, unselected and came from all economic groups as well as from different racial and national aggregates within metropolitan New York. Therefore, although not specifically determined, it is reasonable to assume considerable differences in dietary habits. If, as seems likely, this also implies differences in the composition of the fat intake, then active physiological regulation of the adipose tissue triglyceride composition must exist. Cellular control mechanisms for maintaining the constancy of the adipose tissue fatty acid composition are not known. One aspect of this may, however, be revealed in the present experiments. The *in vitro* studies with human adipose tissue have demonstrated that the synthesized lipid which appears in the medium is unesterified. These data are in accord with the observations of Reshef, Shafrir and Shapiro who found that in rat adipose tissue, unesterified palmitic acid is transferred from the tissue to the medium (19). In man, Dole (20), Gordon and Cherkes (21), Spitzer and Miller (22), and Laurell (23) have observed changes in the serum free fatty acids under conditions of fat mobilization from adipose tissue which indicate that fatty acids, not triglycerides, are released. The present results also demonstrate that there is less than 1.5 per cent of newly synthesized lipid present as free fatty acid in the cell and the remainder is esterified. In the studies of rat mesenteric adipose tissue, Shapiro, Chowers and Rose found that stearate-1-C^{14} was incorporated into the cellular triglycerides; only a small proportion was present as the free acid (15). From these observations, it is suggested that fatty acid synthesized in the cytoplasm is immediately esterified and stored in the intracellular triglyceride reservoir or transferred to the extracellular fluids This rapid disposition to one or another compartment provides flexibility which may facilitate the maintenance of a constant fatty acid composition.

It has been stated and generally accepted that lipomas do not participate in the body economy when there is a demand for fat mobilization through starvation or inanition. The clinical observation on which this allegation is based has been questioned (24) and the *in vitro* studies which have shown that radioactive lipids formed during incubation do appear in the medium also indicate that the potential mechanisms for mobilization of lipoma lipid exist.

At the outset of this study, several hypotheses were formulated which might characterize the fundamental difference between the lipoma and the normal adipose tissue from which it arises. Thus, a localized accumulation of fat could occur if the cells involved had a smaller than normal capacity to oxidize fatty acids, or if there were impairment in the pathway for the mobilization of fat from the cells, or if there were a greater rate of fat synthesis in the tumor than in the normal adipose tissue. One other possibility considered was that there might be a qualitative difference in the biosynthetic pathway which would lead to the formation of fat with a different chemical composition from that of the normal adipose tissue. It is recognized that *in vivo* there are many factors influencing lipid synthesis which cannot be evaluated in the *in vitro* system. Nevertheless, the finding that the lipomas generally incorporate acetate into fatty acids at a greater rate than the normal adipose tissue from the same subject, whereas there was no consistent difference between these two tissues in the rates of acetate oxidation to CO_2, the proportion of newly synthesized lipid transferred to the medium, or the fatty acid composition, suggests that a disturbance in lipid synthesis is a major factor leading to fat accumulation in the lipoma.

SUMMARY

1. The rate of acetate-1-C^{14} incorporation *in vitro* into mixed lipids of lipomas and normal human subcutaneous adipose tissue from the same subject was greater in the tumor than in the normal tissue in 18 of 20 experiments. No significant difference in the rate of acetate-1-C^{14} incorporation into $C^{14}O_2$ or in the proportion of isotopically la-

beled lipid transferred to the medium was found between the two types of adipose tissue.

2. Marked variation was observed in the rate of acetate incorporation into mixed lipids by subcutaneous adipose tissue among the 20 subjects studied. This could not be correlated with age, sex or race.

3. In contrast to the variability of lipid synthesis as measured by acetate incorporation into the mixed lipids, no significant variation was found in the fatty acid composition of the subcutaneous fat between subjects or between the normal adipose tissue fat and that of lipomas.

4. Studies of the distribution of radioactivity in lipid components of adipose tissue following incubation with acetate-1-C^{14} demonstrated about 1 per cent of the total in the tissue free fatty acids and the remainder in the triglycerides. Eighty per cent of the triglyceride radioactivity was recovered in the fatty acids. Similar studies of the lipids in the medium showed recovery of about 90 per cent of the radioactivity in the free fatty acids and the remainder in triglycerides. The rate of accumulation of C^{14} was greater in the saturated fatty acids than in the unsaturated acids in the normal adipose tissue and lipoma.

REFERENCES

1. Wertheimer, E., and Shapiro, B. The physiology of adipose tissue. Physiol. Rev. 1948, **28**, 451.
2. Shapiro, B., and Wertheimer, E . The metabolic activity of adipose tissue. A review. Metabolism 1956, **5**, 79.
3. Hausberger, F. X., Milstein, S. W., and Rutman, R. J. The influence of insulin on glucose utilization in adipose and hepatic tissues in vitro. J. biol. Chem. 1954, **208**, 431.
4. Winegrad, A. I., and Renold, A. E. Studies on rat adipose tissue *in vitro*. I. Effects of insulin on metabolism of glucose, pyruvate and acetate. J. biol. Chem. 1958, **233**, 267.
5. Ball, E. G., Martin, D. B., and Cooper, O. Studies on the metabolism of adipose tissue. I. Effect of insulin on glucose utlization as measured by manometric determination of carbon dioxide output. J. biol. Chem. 1959, **234**, 774.
6. Colowick, S. P., and Kaplan, N. O. Methods in Enzymology. New York, Academic Press, 1955, vol. 1, p. 5.
7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Minneapolis, Burgess, 1945.
8. Davidson, J. D., and Feigelson, P. Practical aspects of internal-sample liquid-scintillation counting. Int. J. appl. Radiat. 1957, **2**, 1.
9. Folch, J., Lees, M., and Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. J. biol. Chem. 1957, **226**, 497.
10. Borgström, B. Investigation on lipid separation methods. III. Separation of tri-, di-, 1-mono- and 2-mono-glycerides. Acta physiol. scand. 1954, **30**, 231.
11. James, A. T., and Martin, A. J. P. Gas-liquid chromatography: The separation and identification of the methyl esters of saturated and unsaturated acids from formic acid to *n*-octadecanoic acid. Biochem. J. 1956, **63**, 144.
12. Lipsky, S. R., Landowne, R. A., and Godet, M. R. The effects of varying the chemical composition of the stationary liquid on the resolution of the long chain saturated and unsaturated fatty acid esters by gas-liquid chromatography. Biochim. biophys. Acta 1959, **31**, 336.
13. Craig, B. M., and Murty, N. L. The separation of saturated and unsaturated fatty acid esters by a gas-liquid chromatography. Canad. J. Chem. 1958, **36**, 1297.
14. Karmen, A. Gas chromatography and lipid metabolism. Amer. Heart J. 1960, **59**, 937.
15. Shapiro, B., Chowers, I., and Rose, G. Fatty acid uptake and esterification in adipose tissue. Biochim. biophys. Acta 1957, **23**, 115.
16. Marks, P. A., Gellhorn, A., and Kidson, C. Lipid synthesis in human leukocytes, platelets, and erythrocytes. J. biol. Chem. 1960, **235**, 2579.
17. Moore, C. H., and Cook, R. P. Human adipose tissue (abstract). Biochem. J. 1959, **73**, 43p.
18. Hirsch, J., Farquhar, J. W., Ahrens, E. H., Jr., Peterson, M. L., and Stoffel, W. Studies of adipose tissue in man: A microtechnic for sampling and analysis. Amer. J. clin. Nutr. 1960, **8**, 499.
19. Reshef, L., Shafrir, E., and Shapiro, B. *In vitro* release of unesterified fatty acids by adipose tissue. Metabolism 1958, **7**, 723.
20. Dole, V. P. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. clin. Invest. 1956, **35**, 150.
21. Gordon, R. S., Jr., and Cherkes, A. Unesterified fatty acid in human blood plasma. J. clin. Invest. 1956, **35**, 206.
22. Spitzer, J. J., and Miller, H. I. Unesterified fatty acids and lipid transport in dogs. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 124.
23. Laurell, S. Plasma free fatty acids in diabetic acidosis and starvation. Scand. J. clin. Lab. Invest. 1956, **8**, 81.
24. Wells, H. G. Adipose tissue, a neglected subject. J. Amer. med. Ass. 1940, **114**, 2177.

THE EFFECTS OF AGING AND DEGENERATIVE VASCULAR DISEASE ON THE MEASUREMENT OF ARTERIAL RIGIDITY IN MAN *

By FRANCOIS M. ABBOUD † AND JOHN H. HUSTON

(From the Cardiovascular Section, Department of Medicine, Marquette University School of Medicine and the Milwaukee County Hospital, Milwaukee, Wis.)

(Submitted for publication December 6, 1960; accepted February 9, 1961)

In 1937, Hallock and Benson demonstrated a progressive loss of aortic distensibility with aging (1). Their observations on the pressure-volume relationship in segments of human aortas from five age groups are shown in Figure 1. The curves which represent the less distensible older aortas have a greater slope (d P/d V). The slope of each curve is not fixed, however, and it changes by bending toward the pressure axis as pressure increases. The rate of this change in slope (or the curvature) also is greater in the curves of older aortas.

The present report deals with an indirect clinical index of the curvature of arterial pressure-volume curves, using the ratio of the change in pulse pressure to the change in diastolic pressure following the inhalation of amyl nitrite. This index is referred to, arbitrarily, as one of "arterial rigidity." A study of Hallock and Benson's curves made it apparent to us that the change in the slope of each curve is associated with a corresponding change in pulse pressure at different diastolic pressures. If the pressure-volume curve is relatively straight, as it is in the young, the curvature and the index will be near zero (Figure 2). If the line curves greatly toward the pressure axis, the index will be positive and large (Figure 3). If it curves the other way, the index will be negative. *In vitro* indices were derived from each of the five aortic distensibility curves and were found to correlate with age (Figure 4). This observation suggested that the values of this index in "normal" subjects also should increase with chronological age. The present investigation was undertaken to test this hypothesis.

* Supported by a research grant from the American Heart Association.

† Work done during tenure of an American Heart Association Research Fellowship. Present address: Cardiovascular Research Laboratories, College of Medicine, State University of Iowa, Iowa City, Iowa.

MATERIALS AND METHODS

Selection of subjects. The subjects for this investigation were patients from the wards and members of the professional staff of Milwaukee County Hospital. One hundred fifteen subjects varying from 8 to 95 years of age were studied. They were separated into nine age groups. Seven subjects, aged 8 to 19 years, comprised the first group; 4 subjects were 20 to 29 years of age; 18 were 30 to 39 years; 24 were 40 to 49 years; 21 were 50 to 59 years; 13 were 60 to 69 years; 16 were 70 to 79 years; 9 were 80 to 89 years; and 3 were 90 to 95 years. A careful clinical examination and review of the hospital records disclosed normal retinal vessels,[1] "normal" blood pressures [2] and freedom from heart disease, overt periph-

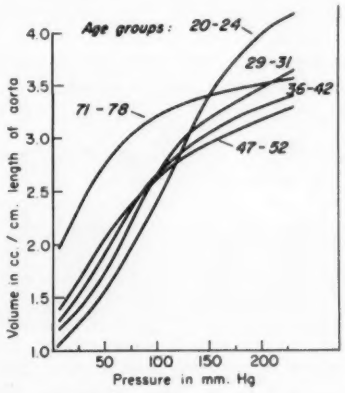


FIG. 1. PRESSURE-VOLUME CURVES OF SEGMENTS OF HUMAN AORTAS FROM FIVE AGE GROUPS (FROM THE EXPERIMENTAL DATA OF HALLOCK AND BENSON) (1). The curve which represents the older aorta has a greater slope (d P/d V). It also bends toward the pressure axis with a greater curvature (in the operating pressure range) than do the curves of younger aortas. (Permission to reprint this figure from the J. clin. Invest. 1937, **16**, 599 was granted by the editor.)

[1] Silver wiring and tortuosity were accepted as normal retinal changes in subjects over 60 years of age.

[2] All the subjects under 60 years of age had blood pressures below 140/80 and all those over 60 years had blood pressures below 170/90.

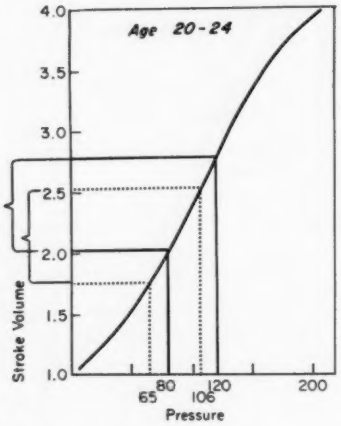


FIG. 2. PRESSURE-VOLUME CURVE OF HUMAN AORTA (AGE 20 TO 24 YEARS) FROM FIGURE 1. Two equivalent stroke outputs were plotted on the ordinate so that their corresponding pulse pressures on the abscissa were within the normal range for the age group and the difference between their diastolic pressures was approximately 15 mm Hg. Note that because the pressure-volume curve is relatively straight there is little change in pulse pressure for the same stroke volume at various pressure levels. The calculation of the rigidity index (fall in pulse pressure $\times$ 100/fall in diastolic pressure) reveals a slight increase in pulse pressure at a lower diastolic level resulting in a small negative value of -7.

eral vascular disease and diabetes. None had a family history of diabetes. Roentgenographic examinations of their chests were normal except for calcification or tortuosity of the aorta in 6 of 28 persons over 70 years of age. Electrocardiograms revealed no significant abnormalities. This group constituted the "normal" subjects used to determine the effect of aging on arterial rigidity.

Twenty-five other patients were selected because of clinical evidence of arteriosclerotic vascular disease. An attempt was made to correlate their arterial rigidity indices with various overt manifestations of arteriosclerosis. Eight of them had intermittent claudication (arteriosclerosis obliterans); 5 had had cerebrovascular accidents; 5 had had myocardial infarctions; 3 appeared prematurely senile and had angina pectoris; 2 were myxedematous; 1 had an aortic arch syndrome with a hard nodular carotid artery; and 1 had a calcified aortic stenosis.

Method. The arterial rigidity index was determined in all subjects. The method was originally described by Conway and Smith (2) and its theoretical background has been discussed. It requires accurate measurement of the blood pressure changes immediately following amyl nitrite inhalation. To be valid it requires a nearly constant left ventricular stroke output during the blood pressure measurement. There is evidence that only an insignificant change in stroke output occurs during the brief period of hypotension which follows the inhalation of amyl nitrite and precedes the onset of reflex tachycardia (2–4). The ratio of the change in pulse pressure times 100 to the related decrease in diastolic pressure during this period gives the index of arterial rigidity. In older subjects with more rigid arteries there was a greater fall in pulse pressure and consequently a higher index.

The nature and purpose of the procedure were discussed with the subjects. They received no premedication and were allowed to rest comfortably on their backs for 10 minutes before the test. Under local infiltration anesthesia, a Cournand needle was introduced into the brachial artery and connected with a short piece of polyethylene tubing (internal diameter, 1 mm) to a strain gage pressure transducer. The resulting signal was amplified and recorded [3] at a paper speed of 25 mm per second. The transducer-recorder system was calibrated against known static pressures and adjusted to give maximal deflections for each blood pressure recording. The systemic arterial pressure, electrocardiogram, and respiratory movements were recorded simultaneously. Respiratory movements were recorded by fitting a hollow rubber tube around the chest and attaching it to a pressure transducer. After establishing control values, a crushed vial of amyl nitrite was held just under the subject's nose during 1 or 2 normal inspirations and he or she was instructed to maintain a constant respiratory pattern without talking, coughing, or sniffing the vapor. Analysis of the pressure tracings involved selec-

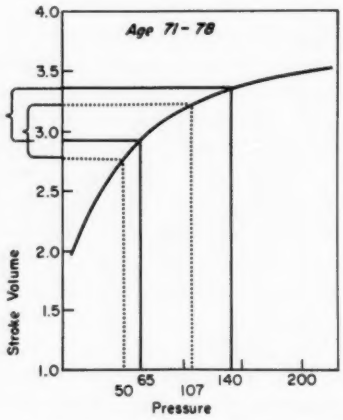


FIG. 3. PRESSURE-VOLUME CURVE OF HUMAN AORTA (AGE 71 TO 78 YEARS) FROM FIGURE 1. Because the slope bends greatly toward the pressure axis as the pressure is increased, there is a marked drop in pulse pressure for the same stroke volume at a lower diastolic pressure. The rigidity index, calculated as described in Figure 2, was + 120.

[3] Sanborn multiple-channel recorder.

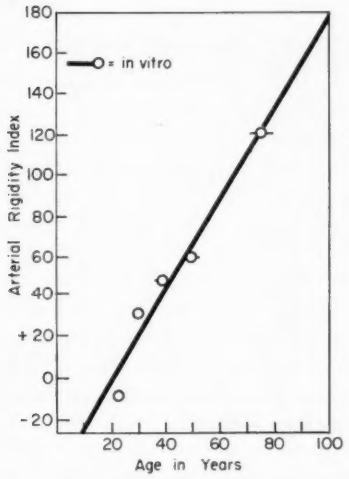


FIG. 4. *IN VITRO* ARTERIAL RIGIDITY INDICES CALCULATED FROM EACH OF THE FIVE PRESSURE VOLUME CURVES OF HALLOCK AND BENSON USING THE METHOD DESCRIBED IN FIGURE 2. Note the progressive increase of these indices with age ($R = 0.977$, $p = < 0.005$).

tion of equivalent beats in respect to heart rate and phase of respiration before the inhalation of amyl nitrite and immediately after its hypotensive effect. Seven to 10 beats usually were available for study before reflex tachycardia occurred (Figure 5). A decrease in diastolic pressure greater than 7 mm Hg was judged arbitrarily to be necessary for a sufficiently accurate calculation. Consistent results were obtained in each of three subjects in whom the test was repeated during a second session. The regression and correlation of the indices with aging were determined (6). An equation for the upper limit of normal was calculated at $+2$ sample standard deviations from the regression.

Rigidity indices also were calculated from five *in vitro* aortic pressure-volume curves of Hallock and Benson (Figure 1). These curves were derived from experimental observations made on isolated segments of thoracic aortas from persons in the age groups 20 to 24, 29 to 31, 36 to 42, 47 to 52 and 71 to 78 years. As far as could be determined, all individuals but those of the last group were normal. Those beyond 70 years showed a minimal degree of coronary disease. Figures 2 and 3 illustrate the method used to calculate the indices from the curves. Two equivalent stroke outputs were plotted on the ordinate so that their corresponding pulse pressures were within the normal range for the age group and the difference between their diastolic pressures was approximately 15 mm Hg. The indices were calculated as they were *in vivo*. The regression and correlation of the *in vitro* indices with age were calculated and compared with the *in vivo* data.

RESULTS

Effect of chronological aging on the arterial rigidity index. The mean arterial rigidity indices for the "normal" groups according to increasing age were: -10.7, $+1.5$, $+17.5$, $+42.5$, $+53.5$, $+99.6$, $+112.6$, $+141.6$, and $+121.0$ (Table I). Six subjects in the first group (8 to 19 years) had an increase in the pulse pressure as the diastolic pressure fell and the mean arterial rigidity was thus expressed with a negative sign. A significant positive correlation ($R = 0.937$, $p = < 0.001$) between the indices and aging was obtained. Fig-

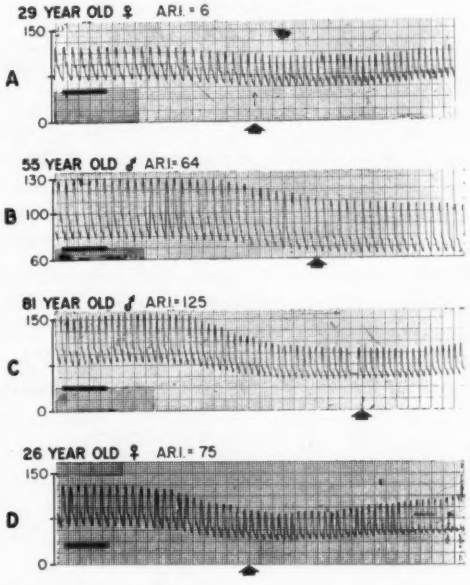


FIG. 5. BRACHIAL ARTERIAL PRESSURES RECORDED BEFORE, DURING AND AFTER AMYL NITRITE INHALATION IN FOUR SUBJECTS. The time of inhalation of amyl nitrite is indicated by the horizontal lines below the tracings. The vertical arrows indicate the onset of reflex tachycardia. A brief period of hypotension precedes the onset of reflex tachycardia. Pressures from this period were used to calculate the arterial rigidity index, A.R.I. Note that the fall in pulse pressure which is insignificant in the young individual (A), progresses with age resulting in higher indices in (B) and (C). A young patient (D) with premature arteriosclerosis had a pressure response comparable to that of older subjects. Note the disappearance of the dicrotic notch in the older subject (C) and in the prematurely arteriosclerotic subject (D). This coincides with the abnormality in dicrotic contours observed by Lax, Feinberg and Cohen (5).

TABLE I

Effect of amyl nitrite inhalation on the blood pressure of 115 "normal" subjects and the calculated mean arterial rigidity index in each age group *

Age groups	No. of subjects and Sex	Average brachial artery pressures				Average change in PP after nitrite	Average rigidity index†
		Before nitrite		After nitrite			
		SP	DP	SP	DP		
yrs		*mm Hg*		*mm Hg*		*mm Hg*	
8–19	7 (5 M)	111.8	65.8	98.5	50.9	+ 1.6‡	− 10.7‡
20–29	4 (2 M)	127.2	77.0	113.3	63.3	− 0.2	+ 1.5
30–39	18 (9 M)	124.5	70.6	108.9	57.3	− 2.3	+ 17.5
40–49	24 (15 M)	130.6	70.0	111.5	56.6	− 5.7	+ 42.5
50–59	21 (15 M)	131.1	70.8	111.0	57.7	− 7.0	+ 53.5
60–69	13 (9 M)	142.3	72.0	120.1	60.9	−11.1	+ 99.6
70–79	16 (11 M)	143.0	66.7	114.3	53.2	−15.2	+112.6
80–89	9 (5 M)	146.8	61.5	115.4	48.5	−18.4	+141.6
90–95	3 (2 M)	167.1	75.0	128.4	57.5	−21.2	+121.0

* M = male; SP = systolic pressure; DP = diastolic pressure; PP = pulse pressure.

† Arterial rigidity index = $\frac{\text{fall in pulse pressure} \times 100}{\text{fall in diastolic pressure}}$.

‡ There was an increase in pulse pressure as the diastolic pressure fell in this group only and the index was expressed with a negative sign.

ure 6 shows the scatter of the individual observations around the regression line which was drawn from the calculated equation: index = (2.19 × age) − 53.84. The sample standard deviation from this regression was ± 16.16 ($p = < 0.001$) and the equation of the upper limit was obtained by adding 2 standard deviations to the regression, i.e., index = (2.19 × age) − 21.52. The regression coefficient was 2.19 ± 0.15 ($p = < 0.001$).

Arterial rigidity indices calculated from in vitro pressure-volume curves of normal human aortas. The indices, calculated from the five curves in Figure 1, according to increasing age were − 10, + 33, + 49, + 60 and + 120, respectively. Figure 4 shows the strong correlation ($R = 0.977$, $p = < 0.005$) between the indices and age with the regression line drawn according to the calculated equation: index = (2.26 × age) − 46.78 ± 11.51 ($p = < 0.005$). The regression coefficient was 2.26 ± 0.28 ($p = < 0.005$).

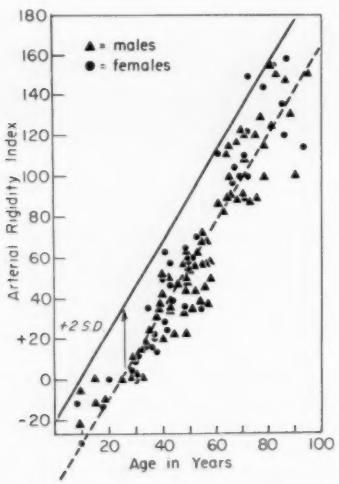


FIG. 6. ARTERIAL RIGIDITY INDICES (ORDINATE) OF 115 "NORMAL" SUBJECTS RANGING FROM 8 TO 95 YEARS OF AGE. The upper limit of normal (solid line) is drawn at + 2 SD from the regression line (broken line).

Comparison between in vivo and in vitro data. Figure 7 shows the close agreement between the mean indices determined clinically and those derived *in vitro*. The regressions of the two groups of data with age were very similar as were their regression coefficients.

Arterial rigidity indices in patients with clinical evidence of arteriosclerosis. The 25 patients in this group had signs and symptoms of arteriosclerotic vascular disease involving various portions of their arterial system. Twenty-three of them had indices that were greater than the upper limits of normal for their age (Figure 8). The usual clinical manifestations of arteriosclerotic vascular disease (advanced arteriosclerotic retinal changes, calcifications or tortuosity of the aorta, and systolic hypertension[4]) were found in only 17 of these 23 patients.

[4] A systolic hypertension was considered present when the diastolic pressure was normal and the pulse pressure was 100 mm Hg or more.

DISCUSSION

Conway and Smith described the ratio of the fall in pulse pressure to the fall in diastolic pressure following the inhalation of amyl nitrite as an index of "arterial elasticity" (2). In a study of aging of the arteries in relation to hypertension (7), they found that the lack of "arterial elasticity" in a group of older hypertensive subjects significantly exceeded that in 16 young "normal" medical students and concluded that hardening of the arteries may be one cause for diastolic hypertension. Because of the well known correlation of arteriosclerosis with chronological aging, we suspected that a number of the hypertensives in their studies might be considered to have "normal elasticity" when compared with "normal" subjects of their same age. A clear understanding of what this index represents and a knowledge of the effect of aging on its values are needed.

A close study of Hallock and Benson's pressure-volume curves indicated that the index is not measuring the slope of these curves but the rate of change of the slope (or the curvature) with pressure. Thus, it is not directly related to the distensibility of the large arteries but it represents the differential of their stiffness [5] with respect to pressure. Its usefulness, in spite of its indirectness, results entirely from the empiric fact that the classical curves of Hallock and Benson show a progressive increase in their curvature with age. This relationship between the index and the curvature brings up three important possibilities. The first is that the index should increase with age since the "curvature" increases with age. This is demonstrated both *in vivo* and *in vitro* in this report. The second possibility is that the index provides an indirect estimate of the number and state of collagenous and elastin fibers in the large arteries. Roach and Burton showed that the shape of arterial pressure-volume curves results from the combined effect of the elastin and collagenous fibers in the vessel wall (9). They proposed that the increased curvature seen in the distensibility curves of old arteries represents an increase in collagenous material and a decrease in

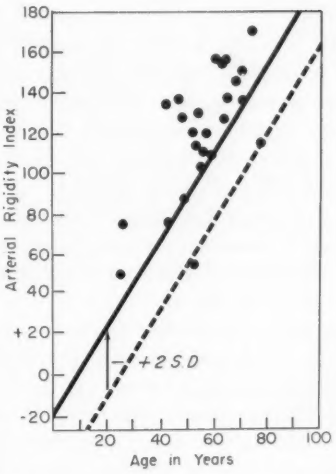


FIG. 8. ARTERIAL RIGIDITY INDICES (ORDINATE) OF 25 PATIENTS WITH CLINICAL EVIDENCE OF ARTERIOSCLEROSIS INVOLVING VARIOUS PORTIONS OF THE ARTERIAL SYSTEM. The solid line represents the upper limit of normal as in Figure 6. Note that 23 arteriosclerotic individuals had arterial rigidity indices which exceeded the upper limit of normal for their age group.

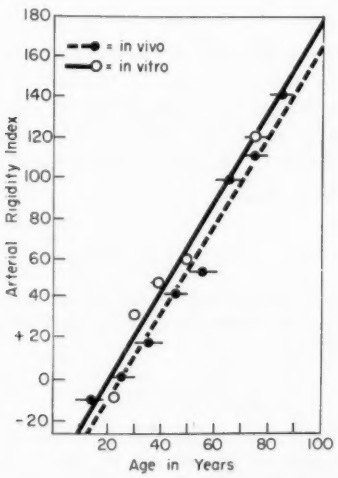


FIG. 7. NOTE THE CLOSE AGREEMENT BETWEEN THE *IN VITRO* INDICES (OPEN CIRCLES) AND THE MEAN VALUES OBTAINED WITH THE AMYL NITRITE TEST IN 115 "NORMAL" SUBJECTS (DOTS). The regression equations and regression coefficients of the 2 groups of data (*in vitro* and *in vivo*) were very similar.

[5] Stiffness (sometimes defined as a secant modulus) has been used to describe behavior of biological materials without reference to cross sectional area and initial length measurements. In this sense stiffness is defined as the ratio of increment in tension to increment in length (8).

the unstretched length of the collagenous fibers as the "slack" in them is taken up, possibly by cross linkages and adhesions (10). The third possibility is that the index may be invalid at pressure levels outside the "normal" range (170 to 55 mm Hg), since the progressive increase in curvature with age occurs only within this pressure range (Figure 1). At pressures higher than 200 mm Hg the curves of the older aortas are straight while those of the younger vessels are still bending toward the pressure axis.

The results obtained in 115 "normal" subjects provide the needed information for a more correct interpretation of the results of the test in patients of all age groups. In order to check the quantitative validity of the *in vivo* "normal" values we compared them with the *in vitro* indices derived from the aortic pressure-volume curves of Hallock and Benson in various age groups. The agreement between the two groups of data was very close (Figure 7). It is apparent from these observations that the method provides a quantitative measurement of the "rigidity" of large arteries.

Our results in 23 of the 25 patients with overt premature arteriosclerosis demonstrate the effect of superimposed degenerative arterial disease on the "rigidity" of aging. The upper limit of normal for this test separates these patients from "normal" individuals of the same age (Figure 8). Only two patients presumed to have arteriosclerosis because of a previous history of myocardial infarction were found to have normal rigidity indices. Such a finding is not surprising, since coronary artery occlusions not uncommonly occur in subjects whose larger arteries do not show significant arteriosclerosis. The older of the two patients was 77 years old and had evidence of aortic tortuosity on the roentgenogram.

Various important applications of this test might be considered. The first is a clinical estimation of the severity of arterial rigidity. Previously, attempts to quantitate this vascular change had been made by measurements of arterial pulse wave velocity (1, 11). This method proved to be of limited clinical value because of the difficulty in measuring the distance over which the pulse wave traveled, especially when arteries were elongated and tortuous. The amyl nitrite test obviates this difficulty. It also may be used to confirm a clinical suspicion of premature arteriosclerosis, particularly in patients who do not yet manifest the usual signs.

As an investigative tool this test might clarify the relationship between arteriosclerosis and the different types of hypertension or the various aspects of the diabetic state. Further, long-term studies of the effect of environmental, dietary and hormonal factors on the "normal" regression of arterial rigidity with age might yield some very useful information.

SUMMARY

A clinical index of arterial rigidity was investgated. In previous studies the upper limit of normal for this index was obtained from the data on young medical students. Because arteriosclerosis progresses with age, the normal values for this index also should increase with age if it reflects arterial rigidity. Our data on 115 "normal" subjects demonstrated a strong positive correlation of this index with age and were in agreement with *in vitro* indices calculated from pressure-volume curves of normal human aortas in similar age groups. An equation representing the upper limit of normal separated the arterial rigidity of aging from that of premature arteriosclerosis. The results suggest that this test offers more than just a qualitative estimate of arteriosclerosis.

With a knowledge of the variation of normal values with age, the applicability of this test can be extended to all age groups and a more correct interpretation of previous studies utilizing this index can be made.

ACKNOWLEDGMENT

The authors wish to thank Dr. John W. Eckstein for his advice and suggestions in the preparation of this manuscript.

REFERENCES

1. Hallock, P., and Benson, I. C. Studies on the elastic properties of human isolated aorta. J. clin. Invest. 1937, **16**, 595.
2. Conway, J., and Smith, K. S. A clinical method of studying the elasticity of large arteries. Brit. Heart J. 1956, **18**, 467.
3. Conway, J. Direct and reflex effects of injections of nitroglycerine and L-noradrenaline in normal and hypertensive rabbits. Clin. Sci. 1955, **14**, 625.

4. Abboud, F., and Huston, J. Unpublished observations.
5. Lax, H., Feinberg, A. W., and Cohen, B. M. Studies of the arterial pulse wave. I. The normal pulse wave and its modification in the presence of human arteriosclerosis. J. chron. Dis. 1956, **3**, 618.
6. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 5th ed. Ames, Iowa, State College Press, 1956, pp. 122–193.
7. Conway, J., and Smith, K. S. Aging of arteries in relation to hypertension. Circulation 1957, **15**, 827.
8. Remington, J. W., Ed. Tissue Elasticity. Washington, American Physiological Society, 1957, p. 197.
9. Roach, M. R., and Burton, A. C. The reason for the shape of the distensibility curves of arteries. Canad. J. Biochem. 1957, **35**, 681.
10. Roach, M. R., and Burton, A. C. The effect of age on the elasticity of human iliac arteries. Canad. J. Biochem. 1959, **37**, 557.
11. Hallock, P. Arterial elasticity in man in relation to age as evaluated by the pulse wave velocity method. Arch. intern. Med. 1934, **54**, 770.

CORRECTION

On page 868 of the article entitled "Sodium Depletion and Renal Conservation of Water" by Geoge P. Baker, Howard Levitin and Franklin H. Epstein (J. clin. Invest. 1961, **40**, 867), the italicized subheading under "Bone Na" in Table II should read *"mEq/g dry solids."* This had been incorrectly printed as *"mEq/100 g dry solids."*

THE SYNTHESIS OF HEME AND GLOBIN IN THE MATURING HUMAN ERYTHROID CELL*

By DAVID G. NATHAN, SERGIO PIOMELLI AND FRANK H. GARDNER WITH THE TECHNICAL ASSISTANCE OF VIRGINIA B. LEMIRE

(From the Richard C. Curtis Hematology Laboratory, Peter Bent Brigham Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

(Submitted for publication December 9, 1960; accepted February 16, 1961)

This report describes observations on the rate of heme and globin synthesis within the maturation period of human marrow erythroid cells. Such studies have been designed to examine the hypothesis that heme and globin are synthesized with temporal independence during erythroid cell maturation and to establish the normal relationship of heme and globin synthesis. With such data it is hoped that further work may lead to a definition of some of the effects of certain hormones and drugs upon heme and globin synthesis and to closer examination of certain refractory anemias with particular emphasis on abnormalities of globin synthesis.

The rates of synthesis of heme and globin within the erythroid cell maturation period are defined in these studies by the daily incremental incorporation of glycine-2-C^{14} into peripheral blood hemin and globin and of Fe^{59} into red cells following intravenous administration of these precursors to four healthy male volunteers.

MATERIALS AND METHODS

I. Subjects. Four male subjects were selected for these studies. Two were patients in the general medical wards of the Peter Bent Brigham Hospital, and 2 were normal healthy members of Mary Knoll Fathers, Jamaica Plain, Mass. Subject 1 (C.A.) was a 58 year old male with evident right carotid insufficiency secondary to atherosclerosis. His peripheral blood values (hematocrit, hemoglobin, white blood cell count and differential) were normal. Subject 2 (A.C.) was a 55 year old male with moderately severe Parkinson's disease treated with Artane (trihexyphenidyl hydrochloride) during the course of the 5-day study. His peripheral blood values were normal. Subject 3 (F.T.) was a 33 year old male in excellent health, whose blood values were normal. Subject 4 (B.A.) was a 45 year old male with mild essential hypertension, whose blood values were normal.

* Supported by the United States Public Health Service Grant A-965 (C4), The Medical Foundation, Inc., The Milton Fund, and the Permanent Charity Fund.

The studies on Subjects 1 and 2 were performed in the hospital, while Subjects 3 and 4 were ambulatory. Diet and fluids were not regulated for any subject.

II. Procedure. Each subject received nearly simultaneous intravenous injections of 100 μc of glycine-2-C^{14} and 5 μc of Fe^{59} as ferrous citrate; the latter was bound either to autologous plasma or to 15 ml of group AB Rh-positive fresh frozen plasma. Fifteen ml of heparinized blood was drawn daily for 7 days from each subject, except from Subject 2 whose blood was drawn for 5 days. Subjects 1 and 2 also received 1.5 g sodium benzoate by mouth 4 and 8 hours after the injection of isotopes, and daily thereafter. Hippuric acid was isolated from a 4-hour urine collection following each dose of sodium benzoate (1).

One ml of each blood sample was counted for Fe^{59} activity in a single-channel pulse-height analyzer with a sodium iodide thallium-activated crystal. Red cell iron uptake was calculated as previously described (1).

The remaining red cells were washed three times with saline, lysed with distilled water, shaken with 2 ml of toluene and allowed to stand overnight at 5° C. The lysate was centrifuged at 700 G to separate lipid and stromal contents from hemoglobin. The hemoglobin solution derived during the first 2 days was dialyzed against running cold water for 12 hours to remove traces of free glycine.

From the hemoglobin solution, hemin was separated by the technique of Labbé and Nishida (2), and globin was separated by a modification of the technique of Rossi Fanelli, Antonini and Caputo (3). The protein was not dialyzed but simply washed copiously with the acetone-HCl mixture. Fe^{59} counts were not found in the protein, and the hemin preparation achieved constant specific radioactivity. The hemin, globin, and an aliquot of the hemoglobin solution were dried *in vacuo* and combusted to $BaCO_3$ by a modification of the Van Slyke-Folch technique (4). Thirty ml of the combustion fluid was added to 50 to 60 mg of hemoglobin or globin and to 10 to 20 mg of hemin. Carrier hemin was occasionally added to improve the yield of $BaCO_3$. Combustion flasks were simmered on heating mantles for 20 minutes and CO_2-free air swept through the flasks into 0.5 M CO_2-free sodium hydroxide. Blank combustions were performed before each series of combustions. The $BaCO_3$ samples were filtered, dried at 110° C for 12 hours and then weighed (weight A). An aliquot of this total yield (weight B) was counted in a liquid scintil-

lation spectrometer (5). The $BaCO_3$ derived from heating of the combustion fluid alone (blank combustion) was determined (weight C). The amount of $BaCO_3$ in the counted sample which was utilized for the calculation of specific activity was considered equal to B − C (B/A − C). In practice this correction for blank combustion was very small. The yields of $BaCO_3$ were 85 to 100 per cent of theoretical yields from hemin, globin and hemoglobin combustion. No self-absorption was detected below 700 mg $BaCO_3$ with this equipment. Hippuric acid was isolated, combusted, and counted to evaluate the rate of turnover of hepatic glycine.

From certain globin samples, glycine was isolated by the technique of Moore and Stein (6). This material (after addition of carrier glycine) was combusted to $BaCO_3$ and similarly counted.

III. Calculations. The theoretical yields of $BaCO_3$ from 100 per cent combustion of 1 mg of hemoglobin, globin, and hemin are 8.3, 8.4, and 10.1 mg, respectively. Since repeated combustion of single samples produced satisfactory reproducibility of specific activity, the specific activity (counts per minute per milligram) of each $BaCO_3$ sample was multiplied by the appropriate figure to derive the specific activity of hemoglobin, globin and hemin. The millimolar specific activity of glycine in globin (globin glycine) and in hemin (hemin glycine) was derived by dividing the globin value by 0.043 (milligrams of glycine in 1 mg of globin) (7, 8) and multiplying by 75 (molecular weight of glycine), and by multiplying the hemin value by 652 (molecular weight of hemin) and dividing by 8 (number of millimoles of glycine in 1 mmole of hemin). The cumulative hemin and globin glycine specific activities were plotted for each day and a line drawn by inspection to connect the points. From these data and the iron uptake curve, the daily ratios of Fe^{59} specific activity and of hemin glycine specific activity to globin glycine specific activity were calculated and plotted. The daily increment of hemin and globin glycine specific activities was calculated from the cumulative hemin and globin curves and separately plotted. The theoretical hemoglobin specific activity was calculated from the addition of 4 per cent of the hemin specific activity and 96 per cent of the globin specific activity. This value was compared to the actual hemoglobin specific activity to evaluate the reproducibility of combustion technique and the thoroughness of the hemin and globin separations.

RESULTS

The data are summarized in Figures 1 and 2. Each figure represents two studies and contains five sections for each study. The shapes of the iron incorporation curves would indicate minimal red cell maturation times of between 5 and 6 days.

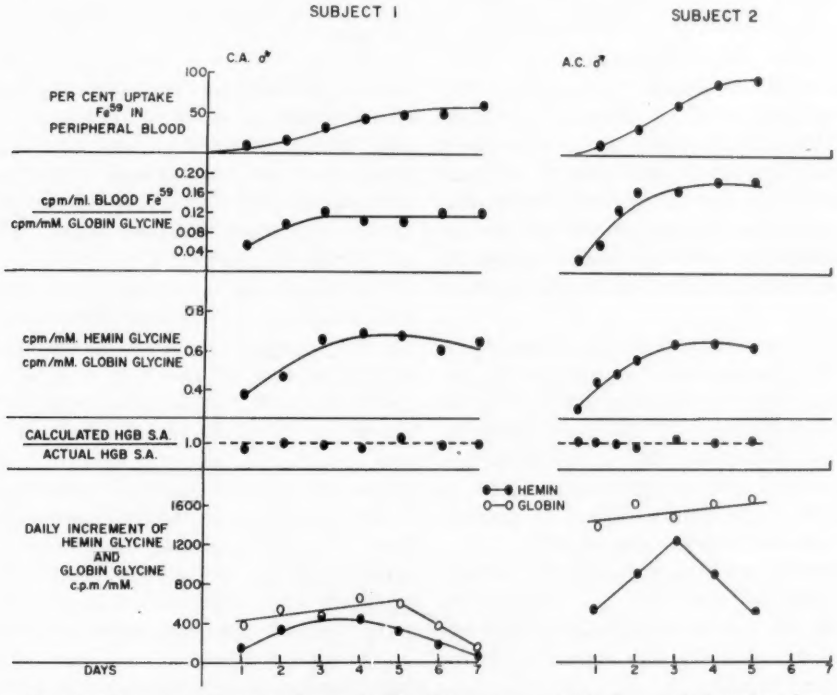


FIG. 1. DATA FROM SUBJECTS 1 AND 2. The data are presented in five vertical sections for each subject (see text).

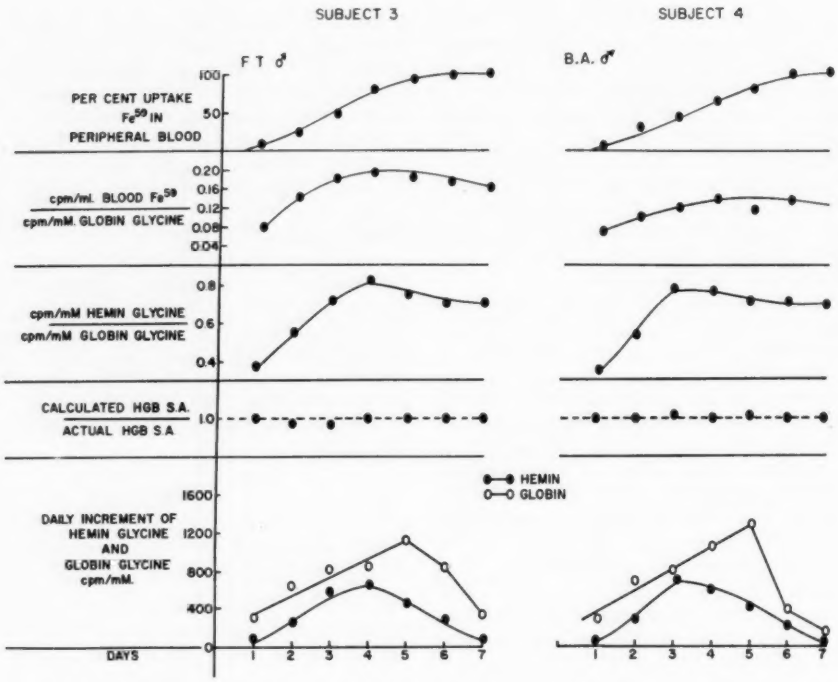


FIG. 2. DATA FROM SUBJECTS 3 AND 4. The data are presented in five vertical sections for each subject (see text).

The curves representing the ratios of the Fe^{59} (cpm per ml blood) to the globin glycine (cpm per mmole) and the hemin glycine (cpm per mmole) to the globin glycine (cpm per mmole) are quite similar and indicate that the time required *in vivo* for synthesis of hemin from glycine and for insertion of iron into hemin are similar enough in normal males to be difficult to distinguish by these techniques.

The curve representing the ratio of hemin glycine to globin glycine would indicate that the initial cells emerging from the marrow following administration of glycine-2-C^{14} are more heavily labeled in the globin moiety than in the heme. As the days proceed, cells emerge with progressive increase of the ratio until a plateau is reached, which in each case is below that of unity.

Comparison of the hemoglobin specific activity calculated from the hemin and globin specific activities with the actual hemoglobin specific activities indicates that the data appear to be reproducible and reliable.

The glycine isolated from globin samples of the first, second and tenth day following glycine-2-C^{14} administration to Subject 1 had specific activities equal to 90, 88 and 87 per cent of the calculated specific activities, respectively. This was probably due to some C^{14}-labeling of the protein by amino acids which had been labeled by transfer of C^{14} from the glycine-2-C^{14}.

The incremental hemin and globin glycine specific activity curves lend more information regarding the timing of hemin and globin synthesis in maturation than do the daily cumulative hemin glycine to globin glycine ratio curves. The former curves are, in effect, mirror images of the marrow relationships, since the cells which emerged on the first day were among the most mature cells in the marrow at the time of isotope administration. The cells appearing on the fifth day were probably among the most primitive cells of the erythroid series at the time of isotope administration. The cells appearing in the peripheral blood on the sixth and seventh days were not likely to have been in a line of progressive maturation at the time of the isotope introduction, but rather began

their maturation when the marrow glycine was of relatively low specific activity following the introduction of the high specific activity "pulse."

The erythroid cells, which were primitive at the time of the radioactive glycine "pulse," appeared in the peripheral blood on the fifth day and had incorporated glycine into globin actively. There is then progressive diminution of this uptake as the cell matures. The changes in hemin specific activity are qualitatively different with a rather bell-shaped curve of glycine uptake and a peak at 3 to 4 days. The curves are not identical, but are very similar in each case. The globin curves decline more steeply in Subjects 3 and 4 than in Subjects 1 and 2. The daily globin increment is greater than the hemin increment but approximates the hemin increment at the hemin peak. There is variability in the approximation from subject to subject. These data suggest that hemin and globin are synthesized in variable proportions depending on the stage of cell maturation.

Figure 3 portrays a representative hippuric acid specific activity curve following the administration of glycine-2-C^{14} to Subject 1. Of note is the fast clearance of the body burden of radioactive glycine, implying a rapid turnover of glycine in the liver. Hippuric acid specific activity extrapolated to zero time does not fairly represent the peak specific activity of liver glycine, since the rate of decline within the first hour or even the first 4 hours is not measured. The data of Nemer, Wise, Washington and Elwyn (9) regarding the rate of decline of the specific activity of hepatic L-serine following the administration of L-serine-3-C^{14} to rats show that the most abrupt change is measured within the first several minutes after injection.

DISCUSSION

Numerous studies have been performed to elucidate the peak synthetic rates of heme and globin during the maturation of the erythroid cell. The early data of Thorell (10) and the experiments of Muir, Neuberger and Perrone (11), Altman and associates (12) and Kassenaar, Morell and London (13), some of whom have used quite different approaches to the problem, lend credence to the thesis that a temporal dissociation of the synthetic rates of these compounds exists. However, species differences are noted, and the *in vitro* studies of Kruh and Borsook (14), and of Morell, Savoie and London (15), do not support this impression. There is little information regarding human physiology in this regard, although the recent work in humans of Bannerman, Grinstein, Moore and Varva (16, 17) would suggest a dissociated mechanism.

The technical approach to the problem adopted in this report involves the measurement of the hemin and globin specific activity of the peripheral blood following the introduction of a "pulse" of glycine-2-C^{14} and Fe^{59}. The increment of carbon specific activity in the two compounds is thought to represent the uptake of the isotope into the products in each of the maturing erythroid cells of marrow. These cells mature and emerge from the marrow as reticulocytes with final heme and globin specific activities that are direct functions

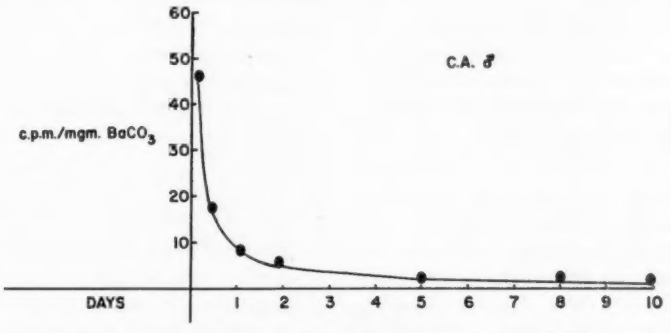


FIG. 3. URINARY HIPPURIC ACID SPECIFIC ACTIVITY FOLLOWING INTRAVENOUS GLYCINE-2-C^{14} ADMINISTRATION TO SUBJECT 1. The results are expressed as the specific activity of $BaCO_3$.

of their uptake of the "pulse" of precursor and their previous or subsequent synthesis of heme and globin from nonradioactive precursor glycine.

The ranges of synthetic rates of each compound as a function of maturation may then be assessed from the incremental curves with the following assumptions. *1*) The intracellular precursor pools of glycine for heme and globin are of equal specific activity in each cell. *2*) The radioactivity of the glycine in the marrow rapidly achieves a high concentration and falls swiftly to low levels so that persistent labeling of the cells not exposed to the high "pulse" contributes very little to the incremental curve. *3*) No appreciable amounts of intermediate compounds, which exist between glycine and the products under scrutiny, are present in concentrations capable of diluting the precursor for one compound in excess of the other. *4*) There must be no appreciable turnover or re-utilization of parts of one product in excess of the other. *5*) Net synthesis of the products must be shown to occur. These stipulations have recently been carefully reviewed by Russell (18).

Some, but not all, of the above assumptions have been evaluated during these experiments. *1*) No information regarding possible separate pools of glycine for heme and globin synthesis in human erythroid cells is available. Recent evidence has been presented (19) to indicate that different human tissues maintain varying glycine specific activities following glycine-2-C^{14} administration so that the concept of a single homogeneous body pool of glycine is no longer acceptable. The recent publications of Rabinovitz and Olson (20) and of Kipnis and Reiss (21) indicate a linear uptake of certain radioactive amino acids into protein *in vitro* as though little mixing of the amino acids in an intracellular pool had taken place. *2*) The urinary hippuric acid specific activity curve indicates that the high pulse of radioactivity, at least in the liver, is short. The first value is taken 4 hours after the administration of glycine and greatly underestimates the actual peak specific activity of glycine which is achieved (9). *3*) The problem of intermediate compounds has been approached by assuming that such intermediates are more likely to be detected along the heme rather than the globin pathway. That this assumption may not be valid is best shown by the complex kinetics of reticulocyte protein synthesis described by Rabinovitz and Olson (20). Attempts to bypass the heme intermediates by the use of radioiron showed that the rates of iron and glycine incorporation were quite similar by this technique. However, the work of Allen and Jandl (22) would indicate that this may be fortuitous, since these workers have demonstrated complex kinetics of iron incorporation into heme, including delay of passage of iron through the stroma which causes a lag phase in the appearance of iron in the heme. *4*) Turnover and re-utilization of heme and globin within the marrow has not been established. The well known early stercobilin peak (23) is associated with erythropoiesis, but whether it is due to medullary heme turnover, erythroid cell death (24) or destruction of excess peripheral reticulocytes, is not established. Definite information on globin turnover is lacking. If medullary erythroid cell death is responsible for the early stercobilin peak, heme and globin would be expected to be catabolized or re-utilized at equal rates within the marrow. *5*) Net synthesis of heme and globin by the marrow is obvious, but measurements of peripheral blood specific activity do not take into account the complex processes of cell division (25–27) which accompany maturation throughout the marrow. The heme and globin incremental curves reflect not only the synthetic rates of the compounds but also the number of cells of a given stage of maturation present in the marrow at the time of isotope administration (24).

The above considerations prevent rigid interpretation of these data. The results do suggest, however, that globin and heme are not synthesized at equal rates throughout maturation. It appears that the globin synthetic rate is maximal very early in maturation and that the rate steadly declines during maturation. Heme synthesis appears to be maximal during the mid-portion of maturation and is less intense during the early and late phases.

Some confirmation of these interpretations is available from recent radioautographic studies of human marrow *in vitro*. Gavosto, Maraini and Pileri (28) have shown that the human proerythroblast takes up approximately 15 times the amount of tritiated leucine as does the polychromatophilic erythroblast. Since the bulk of proerythroblasts divide several times before maturation and emergence from the marrow, their final globin specific activities might be expected to be

two or three times that of the cells which emerge 1 day after the "pulse" of glycine-2-C^{14} is given. This type of relationship is observed in the incremental curves. Unfortunately, Fe^{59} radioautographs do not reflect heme synthesis and cannot be used in comparison, since so much of the iron in the early cells appears to be present as ferritin and not as heme (29).

The finding of temporal independence of heme and globin synthesis in human red cell maturation presages the detection of lesions of production of one or the other compound (30). Perhaps the most common of lesions, iron deficiency, may have as its counterpart a refractory anemia due to deficient production of globin. Thalassemia may be a special form of such a syndrome.

The daily and incremental curves not only suggest independence of heme and globin synthesis but also imply that reticulocytes may emerge from the marrow containing concentrations of globin above those consistent with their hemoglobin concentrations, since the cells which emerge on the first day maintain a higher globin than heme specific activity. This state may contribute to the macrocytosis, hypochromia and polychromatophilia of reticulocytes. Reticulocyte maturation may include a process of extirpation of excess globin. Further data are required to evaluate this hypothesis.

SUMMARY

1. An *in vivo* method of measuring the timing in red cell maturation of heme and globin synthesis has been described.

2. The data in four normal patients are presented.

3. The data indicate that globin synthesis commences at a very primitive stage of maturation and continues throughout maturation with a linear decline in synthetic rate. Heme synthesis appears to be most active at a more developed stage of maturation, and the bulk of heme synthesis is confined to a more narrow span of the maturation time.

4. The rates of incorporation of iron and glycine into heme appear nearly equal *in vivo*.

5. The difficulties in interpretation of these data are discussed.

ACKNOWLEDGMENTS

The authors wish to thank Dr. David Elwyn and Dr. David Gitlin for their assistance with chromatographic techniques and Dr. Rudi Schmid for helpful suggestions and manuscript review.

REFERENCES

1. Nathan, D. G., and Berlin, N. I. Studies of the production and life span of erythrocytes in myeloid metaplasia. Blood 1959, **14**, 668.
2. Labbé, R. F., and Nishida, G. A new method of hemin isolation. Biochim. biophys. Acta 1957, **26**, 437.
3. Rossi Fanelli, A., Antonini, E., and Caputo, A. Studies on the structure of hemoglobin. I. Physiocochemical properties of human globin. Biochim. biophys. Acta 1958, **30**, 608.
4. Van Slyke, D. D., Plazin, J., and Weisiger, J. R. Reagents for the Van Slyke-Folch wet carbon combustion. J. biol. Chem. 1951, **191**, 299.
5. Nathan, D. G., Davidson, J. D., Waggoner, J. G., and Berlin, N. I. The counting of barium carbonate in a liquid scintillation spectrometer. J. Lab. clin. Med. 1958, **52**, 915.
6. Moore, S., and Stein, W. H. Chromatography of amino acids on sulfonated polystyrene resins. J. biol. Chem. 1951, **192**, 663.
7. Hill, R. J., and Craig, L. C. Countercurrent distribution studies with adult human hemoglobin. J. Amer. chem. Soc. 1959, **81**, 2272.
8. Stein, W. H., Kunkel, H. G., Cole, R. D., Spackman, D. H., and Moore, S. Observations on the amino acid composition of human hemoglobins. Biochim. biophys. Acta 1957, **24**, 640.
9. Nemer, M. J., Wise, E. M., Jr., Washington, F. M., and Elwyn, D. The rate of turnover of serine and phosphoserine in rat liver. J. biol. Chem. 1960, **235**, 2063.
10. Thorell, B. Cellular formation of intermediates during haemoglobin synthesis *in* Ciba Found. Symp. on Porphyrin Biosynthesis and Metabolism, G. E. W. Wolstenholme and E. C. P. Millar, Eds. Boston, Little, Brown, 1955, p. 174.
11. Muir, H. M., Neuberger, A., and Perrone, J. C. Further isotopic studies on haemoglobin formation in the rat and rabbit. Biochem. J. 1952, **52**, 87.
12. Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K. Hemoglobin synthesis from glycine labeled with radioactive carbon in its α-carbon atom. J. biol. Chem. 1948, **176**, 319.
13. Kassenaar, A., Morell, H., and London, I. M. The incorporation of glycine into globin and the synthesis of heme *in vitro* in duck erythrocytes. J. biol. Chem. 1957, **229**, 423.

14. Kruh, J., and Borsook, H. Hemoglobin synthesis in rabbit reticulocytes *in vitro*. J. biol. Chem. 1956, **220**, 905.
15. Morell, H., Savoie, J. C., and London, I. M. The biosynthesis of heme and the incorporation of glycine into globin in rabbit bone marrow *in vitro*. J. biol. Chem. 1958, **233**, 923.
16. Bannerman, R. M., Grinstein, M., and Moore, C. V. Haemoglobin synthesis in thalassaemia; *in vitro* studies. Brit. J. Haemat. 1959, **5**, 102.
17. Grinstein, M., Bannerman, R. M., Varva, J. D., and Moore, C. V. Hemoglobin metabolism in thalassemia; *in vivo* studies. Amer. J. Med. 1960, **29**, 18.
18. Russell, J. A. The use of isotopic tracers in estimating rates of metabolic reactions. Perspec. Biol. Med. 1958, **1**, 138.
19. Schapira, G., Dreyfus, J. C., Kruh, J., Labie, D., and Padieu, P. Absence of a single metabolic pool of free tissue glycine *in* Proceedings of the Second United Nations International Conference on the Peaceful Uses of Atomic Energy. Geneva, United Nations, 1958, vol 25, part 2, p. 21.
20. Rabinovitz, M., and Olson, M. E. Protein synthesis by rabbit reticulocytes. I. Kinetics of amino acid incorporation *in vitro* into protein fractions of intact cells. J. biol. Chem. 1959, **234**, 2085.
21. Kipnis, D. M., and Reiss, E. The effect of cell structure and growth hormone on protein synthesis in striated muscle (abstract). J. clin. Invest. 1960, **39**, 1002.
22. Allen, D. W., and Jandl, J. H. Kinetics of intracellular iron in rabbit reticulocytes. Blood 1960, **15**, 71.
23. Gray, C. H., and Scott, J. J. The effect of haemorrhage on the incorporation of [α-14C] glycine into stercobilin. Biochem. J. 1959, **71**, 38.
24. Stohlman, F. Observations on the kinetics of red cell proliferation *in* The Kinetics of Cellular Proliferation, F. Stohlman, Ed. New York, Grune and Stratton, 1959, p. 318.
25. Alpen, E. L., and Cranmore, D. Observations on the regulation of erythropoiesis and on cellular dynamics by Fe^{59} autoradiography. *Ibid.*, p. 290.
26. Lajtha, L. G., and Suit, H. D. Uptake of radioactive iron (^{59}Fe) by nucleated red cells *in vitro*. Brit. J. Haemat. 1955, **1**, 55.
27. Suit, H. D., Lajtha, L. G., Oliver, R., and Ellis, F. Studies on the ^{59}Fe uptake by normoblasts and the failure of X-irradiation to affect uptake. Brit. J. Haemat. 1957, **3**, 165.
28. Gavosto, F., Maraini, G., and Pileri, A. Radioautographic investigations on DNA and protein metabolism in 2 cases of Di Guglielmo's disease. Blood 1960, **16**, 1122.
29. Sondhaus, C. A., and Thorell, B. Microspectrophotometric determination of nonheme iron in maturing erythroblasts and its relationship to the endocellular hemoglobin formation. Blood 1960, **16**, 1285.
30. London, I. M. Metabolism of the mammalian erythrocyte. Bull. N. Y. Acad. Med. 1960, **36**, 79.

QUANTITATIVE PAPER CHROMATOGRAPHY OF CONJUGATED 17-KETOSTEROIDS IN PLASMA *

By SUZANNE CONRAD, VIRENDRA MAHESH AND WALTER HERRMANN

(From the Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Conn., and The Department of Endocrinology, Medical College of Georgia, Augusta, Ga.)

(Submitted for publication December 14, 1960; accepted February 16, 1961)

The determination of urinary 17-ketosteroids has limited applications in the understanding and diagnosis of the nature of endocrine disorders associated with mild hirsutism and virilism in the female, because a very large number of these patients excretes 17-ketosteroids well within the normal range. Recent experience of Finkelstein, Forchielli and Dorfman (1) suggests that in these cases the blood levels and nature of the circulating androgens may be very important. Earlier indirect evidence (2–12) and the more recent isolation and partial identification of sulfates of dehydroisoandrosterone, androsterone, and etiocholanolone by Baulieu (13) indicate that 17-ketosteroids are present in plasma mainly in the form of esters. Direct estimation of these conjugated steroids in plasma offers distinct advantages: the alteration of the steroid molecule which occurs with certain types of hydrolysis as well as possible problems of incomplete hydrolysis can be circumvented. Also, information concerning the physiological significance of the steroid conjugating mechanisms may be gained through direct qualitative and quantitative estimation of these conjugates. The present paper describes a convenient method, using quantitative paper chromatography, for the estimation of dehydroisoandrosterone and androsterone sulfates (DHIA-SO_4 and Andro-SO_4) in plasma.

MATERIALS AND METHOD

All reagents and solvents used were of analytical grade, and all solvents, except absolute ethanol, were redistilled prior to use. Florisil was purified as described earlier (14). M-dinitrobenzene was purified by sublimation, using a modification of the "cold finger" technique, and stored in the dark at room temperature.

The sodium salts of dehydroisoandrosterone sulfate (DHIA-SO_4) and androsterone sulfate (Andro-SO_4) were obtained from Mann Research Laboratories, Inc., N. Y., and androsterone glucuronide was supplied through the courtesy of Dr. J. J. Schneider, Jefferson Medical College, Philadelphia, Pa.

Alkaline M-dinitrobenzene reagent (DNB). Potassium hydroxide pellets were dissolved in absolute ethanol by shaking gently. The solution was filtered through a sintered glass funnel under vacuum and adjusted to 3 N with hydrochloric acid prior to use. The DNB reagent was prepared immediately prior to use by mixing the 3 N absolute ethanolic potassium hydroxide with twice its volume of 2 per cent DNB (wt/vol) in absolute ethanol.

Collection of plasma. Blood from fasting subjects was drawn into heparinized syringes between 8:30 and 9:30 a.m. The blood was then transferred to a glass centrifuge bottle containing 1 ml of heparin per 100 ml of blood and centrifuged at 2,500 rpm for 30 minutes. The plasma was removed with a syringe equipped with a long spinal needle and transferred to a clean glass vessel.

Extraction of steroid conjugates. All analyses were run in triplicate. Fifteen-ml aliquots [1] of the plasma were placed in three 250-ml Erlenmeyer flasks and 45 ml of absolute ethanol was added to each flask. The flasks were swirled several times and placed in an ice bath for 10 to 15 minutes. After filtering the mixture, under vacuum, through two discs of Whatman no. 1 filter paper, the flasks were rinsed three times with 10 ml of absolute ethanol, and these washings were then used to wash the precipitate. The filtrate was transferred quantitatively to a 1 L round-bottomed flask with further washings of absolute ethanol and evaporated to near dryness (1 to 2 drops) on a flash evaporator (Laboratory Glass and Instruments Corp., model FE-2) under vacuum, using dry ice in ethylene glycol for refrigeration. Secondary butanol (15 ml) was then added to effect a partial separation of steroid conjugates from inorganic salts. The steroid conjugates, which dissolve quantitatively in secondary butanol, along with small amounts of inorganic salts were transferred to a 300 ml round-bottomed flask and the transfer completed with three 5-ml washings with secondary butanol. The secondary butanol solution was then evaporated to dryness on the flash evaporator and the extract transferred quantitatively to a test tube with five washings of absolute ethanol and dried at 45° to 50° C with a gentle stream of air.

Preliminary purification by column chromatography. Glass columns (1 cm in diameter, 11 cm in height) equipped with a 100 ml reservoir and a ground-glass stop-

* This work was supported by United States Public Health Service Grant no. A2845.

[1] Since completion of this work, the method has been adapted to 10-ml aliquots.

TABLE I

Column chromatography elution scheme

Eluting solvent	Effluent	Fraction no.	17-Ketosteroid content
	ml		
Benzene	20		
Benzene:ethyl acetate (1:1, vol/vol)	10	I	Free 17-ketosteroid, if any
Ethyl acetate	10		
Ethyl acetate:absolute ethanol (9:1, vol/vol)	25		
Ethyl acetate:absolute ethanol (1:9, vol/vol)	25	II	17-Ketosteroid sulfates
Absolute ethanol	20	III	No steroid; impurities only
Absolute ethanol:water (9:1, vol/vol)	10		
Absolute ethanol:water (1:1, vol/vol)	10	IV	17-Ketosteroid glucuronides
Absolute ethanol:water (1:9, vol/vol)	10		
Water	20	V	No steroid content

cock and plugged with a small quantity of glass wool were packed with 4.5 g of activated Florisil in benzene. During the addition of Florisil, the column was tapped with a rubber hammer to assure the escape of air bubbles. The benzene was allowed to run out until the lower edge of its meniscus just touched the surface of the Florisil. The crude extract was transferred quantitatively to the column with three washings (2 drops of absolute ethanol, 3 drops of benzene) and rinsed three times with benzene. The elution was carried out with benzene, benzene-ethyl acetate, ethyl acetate, ethyl acetate-ethanol, ethanol, ethanol-water, and water as shown in Table I. Fraction II, the sulfate fraction, and fraction IV, the glucuronide fraction, were evaporated to dryness under vacuum and the residue transferred quantitatively to test tubes with five 1-ml washings of ethyl acetate: methanol (2:1 vol/vol) and re-evaporated.

Florisil was chosen as the supporting medium in preference to alumina, Celite or silica gel because it gave better separation and recovery of the sulfates and glucuronides.

Paper chromatography. Standards and unknowns were applied to 1-inch strips of Whatman no. 2 chromatography paper. The strips were cut from the same sheet (46 × 57 cm) after a starting line had been pencilled 3 inches from one end. This procedure insured that all compounds would be applied to the same face of the paper and run in the same direction. A mixture of standards containing DHIA-SO_4 and Andro-SO_4 in absolute ethanol was pipetted into test tubes at various concentrations in triplicate and dried. The dried standards and plasma eluates were applied to the strips by rinsing the test tubes three times with 4 drops of ethyl acetate: methanol (2:1, vol/vol), and the compounds concentrated at the starting line by the method of Bush (15), using the same mixture. The strips were air-dried and placed in a paper-lined tank containing Schneider and Lewbart's system no. 2 (16). After equilibration for 3 hours, the mobile phase was added and allowed to run off the strips. The running time varied from 18 to 24 hours. The strips were air-dried, and were then ready for quantification.

Quantification. In quantitative paper chromatography, standardization of all steps involved is mandatory to insure proper reproducibility of the results. The Zimmermann reagent must be freshly prepared, the staining procedure adhered to rigorously, and the drying and heating times, as well as the temperature of the chromatography oven, must be strictly controlled. The humidity of the atmosphere should not exceed 40 per cent.

Separate solutions of KOH and DNB were freshly prepared in absolute ethanol. KOH was filtered through sintered glass under vacuum and adjusted to 3 N with HCl immediately prior to use. The paper strips were stained in groups of four. Fourteen ml of 2 per cent DNB and 7 ml of 3 N KOH were mixed and placed in a porcelain moistener. Each strip was run over the surface of the roller twice, dried horizontally over a fan for 90 seconds, and hung in the chromatography oven at 45° C for 3 minutes. The rolling and horizontal drying assure an even application of the Zimmermann reagent and uniformity of background throughout the entire length of the strips. After heating, the strips were stored between two larger blank strips previously treated with the reagent (17) for 1 hour, the time required for stabilization of the color.

Quantification was carried out in a Spinco model RB Analytrol equipped with a B_2 cam and two 550-mμ interference filters. With the B_2 cam, the Analytrol records a linear plot of the optical densities along the strip being scanned. To determine the optimal wave length for

scanning, the Zimmermann-positive area on a stained strip was cut out, placed in the cell holder of a Beckman DU spectrophotometer, and read against a blank, which had been cut from an area of background from the same strip. It was consistently found that the peak absorption occurred at 565 mμ. The 550-mμ interference filters used provide the closest approximation to this commercially available at present. That the readings at this wave length follow Beers' law is illustrated by the standard curves (Figure 1).

The Zimmermann-positive spots appeared on these strips as light violet areas against a brownish background which varied with the alkalinity of the chromatographic system. To insure optimal scanning, each strip was placed in a carrier of clear heavy plastic which centered the strip over the slit opening. A typical scanning curve is shown in Figure 2.

Standard curves. A standard curve was drawn next. It has been noted that triplicate determinations of the standards and plasma extracts have resulted in better standard curves and more reproducible results. Therefore, the plasma was initially divided into three aliquots, each extracted and chromatographed separately as described above. The standard mixture was chromatographed on paper, in triplicate, at concentrations of 20, 40, 60 and 100 μg for DHIA-SO_4 and at 5, 10, 15 and 25 μg for Andro-SO_4. Using four concentrations assured proper standard curves for quantification of the plasma extract, and all plasma aliquots tested thus far contained the sulfates in amounts within the limits of the above standard concentrations.

The values for each standard strip were calculated as above, and the means of the triplicates of each concentration determined. The individual values and the means were next plotted on graph paper, and the standard curve was drawn. The plasma values were then calculated by reading them from this curve.

RESULTS

A. Recovery of added standards. A number of experiments was done in which DHIA-SO_4 and/or Andro-SO_4 were added to the plasma aliquots. The standards were dissolved in absolute ethanol and added to the plasma before its precipitation with absolute ethanol. The protocol given above was followed, and the results are listed in Table II.

B. Characterization of dehydroisoandrosterone and androsterone sulfates. The compound in the dehydroisoandrosterone sulfate zone from paper chromatograms was eluted using ethyl acetate: methanol (2:1, vol/vol) and evaporated to dryness. It was then dissolved in 10 ml of 0.5 M acetate buffer (sodium acetate: acetic acid, pH 4.8) and incubated for 24 hours at 38° C with 10,000

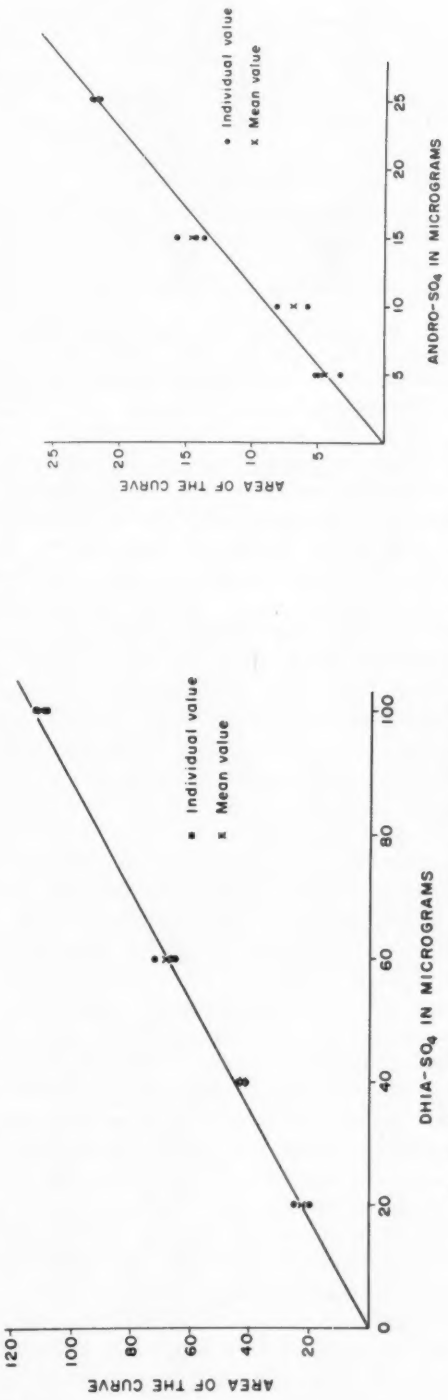


FIG. 1. STANDARD CURVES FOR DEHYDROISOANDROSTERONE SULFATE (LEFT) AND ANDROSTERONE SULFATE (RIGHT).

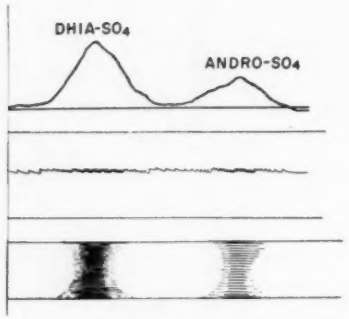


FIG. 2. SCANNING CURVE OF PAPER CHROMATOGRAM. The upper panel is a recording trace; the middle panel is an integrator trace; the lower panel is a facsimile of a paper chromatogram.

U Ketodase (β-glucuronidase). The incubation mixture was extracted with 100 ml ether : ethyl acetate (2:1). The organic layer was washed twice with 10 ml of 1 N NaOH and once with 10 ml of water. The washings were combined with the incubation mixture, which was then brought to pH 1 with dilute hydrochloric acid and continuously extracted with ether for 36 hours.

Aliquots of extracts after β-glucuronidase and HCl hydrolysis were run for 4 and 16 hours on a Whatman no. 2 sheet in 1-inch lanes along with free dehydroisoandrosterone in a system consisting of ligroin : methanol : water (100:96:4, vol/vol) (L/96) designed to separate free 11-deoxy-17-ketosteroids (18). The paper strips were developed using alkaline DNB. The β-glucuronidase hydrolysis product did not show the presence of any 17-ketosteroids, indicating the absence of glucuronides. The HCl hydrolysis product indicated the presence of a 17-ketosteroid which agreed in its color reaction and rate of migration on paper, in the above mentioned system, with dehydroisoandrosterone. A second aliquot of the HCl hydrolysis product was acetylated using acetic anhydride and pyridine and run for 4 hours in a 1 inch lane of Whatman no. 2 paper along with dehydroisoandrosterone acetate in the same system. On staining with alkaline DNB, it was found to be identical in color reaction and rate of migration with dehydroisoandrosterone acetate.

Approximately 40 μg of the HCl hydrolysis product was refluxed for 6 hours with a mixture of 20 ml of acetone, 30 ml of benzene and 0.5 g of aluminum isoporpoxide and the mixture washed twice with 25 ml 1 N H_2SO_4 (19). The organic layer was washed with water, dried over sodium sulfate and evaporated to dryness. It was then run for 4 hours on a 2-inch lane of Whatman no. 2 paper in L/96 along with Δ^4-androstene-3-17-dione. The 2-inch lane was cut into four ½-inch strips: one strip was developed with alkaline DNB while another was tested for NaOH fluorescence. The Oppenauer oxidation product had the same NaOH fluorescence reaction as a Δ^4-3-ketone, the same color with alkaline DNB and rate of migration as Δ^4-androstene-3-17-dione. The steroid zone of the other ½-inch strips was eluted and one fraction was subjected to acetylation, the other to chromic acid oxidation. These two reactions left the steroid molecule unchanged, as revealed by paper chromatography. The various steps in the identification of dehydroisoandrosterone sulfate are given in Figure 3.

The characterization of androsterone sulfate was carried out along the same lines as that of dehydroisoandrosterone although not as extensively, owing to small amounts of available material. The following experimental evidence for proof of identity was found: *1*) failure of hydrolysis with β-glucuronidase; *2*) formation of free androsterone by hydrolysis with HCl at pH 1, identified by paper chromatography; and *3*) acetylation of the androsterone thus produced yielded androsterone acetate which agreed with an authentic sample of androsterone acetate. Further proof of the identity of the sulfate moiety with both DHIA-SO_4 and Andro-SO_4 was obtained with the rhodizonate reaction as described by Schneider and Lewbart (20).

C. Plasma values. DHIA-SO_4 and Andro-SO_4 values were determined in the plasma of normal males between the ages of 20 and 40. These

TABLE II

Recoveries of standards added to the plasma aliquot

DHIA-SO_4		Andro-SO_4	
Added	Recovered	Added	Recovered
μg	%	μg	%
20	100	20	99
		20	91
		15	84
80	100	20	100
		15	100
40	94		

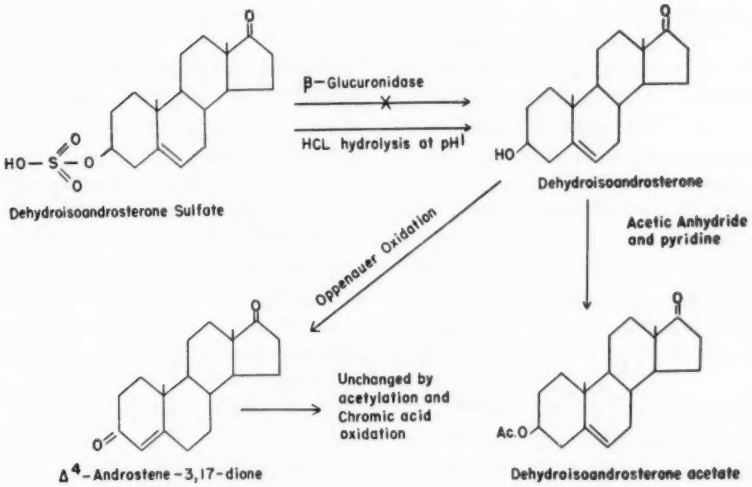


FIG. 3. IDENTIFICATION OF DEHYDROISOANDROSTERONE SULFATE.

values ranged from 155 to 298 μg per 100 ml for DHIA-SO_4 and from 29 to 92 μg per 100 ml for Andro-SO_4 (Table III).

Four plasma samples from normal females in this same age group have also been analyzed. Although this is a very small group, the DHIA-SO_4 levels (range, 85 to 166 μg per 100 ml) seem to be lower than those found in the males, while the Andro-SO_4 levels (range, < 15 to 45 μg per 100 ml) have generally been within the same range as those of the males (Table III).

Plasma drawn preoperatively from a patient with the Stein-Leventhal syndrome was studied. The DHIA-SO_4 level was 342 μg per 100 ml plasma, well above that of any of the normal males studied, while the Andro-SO_4 level was 70 μg per 100 ml. On the other hand, a patient with virilizing Cushing's syndrome secondary to an adrenal tumor (proven at operation), had 185 μg DHIA-SO_4 per 100 ml plasma and 552 μg Andro-SO_4 per 100 ml. This value for DHIA-SO_4 is at the lower level of the male range, and may not be significant; however, the Andro-SO_4 level is about seven times the normal range, and seems highly significant. This latter plasma also contained a third conjugate which has been tentatively identified as etiocholanolone sulfate and is the only plasma analyzed thus far that has been found to contain this particular conjugate in amounts detectable by the present method.

DISCUSSION

The present paper offers essentially two new aspects to the study of 17-ketosteroids in plasma: the direct estimation of these moieties in their conjugated form and the use of quantitative paper chromatography as a routine method.

In approaching the more difficult problem of extraction and purification of the steroid esters from plasma, as compared with a similar procedure following hydrolysis, it was hoped that possible losses incurred during acid treatment could be avoided. It is well known that complete hydrolysis is not achieved with currently available methods, and that dehydroisoandrosterone, par-

TABLE III

Plasma values

DHIA-SO_4		Andro-SO_4	
Subject*		Subject	
	μg %		μg %
WH	176	IM	73
LM	288	OM	64
EM	195	RS	34
T	298	T	51
JE	244	DW	92
SS	162	SS	29
WH	155	WH	39
SC	127	SC	<15
RP	166	RP	<15
MW	85	MW	45
AB	109	AB	25

* SC, RP, MW and AB are females.

ticularly, is partially destroyed by contact with strong mineral acids. These facts must be weighed against the more cumbersome quantitative recovery of such highly polar substances as the 17-ketosteroid sulfates. The figures obtained when the 17-ketosteroid sulfates were added to plasma, as well as the absence of Zimmermann reactive material following the extraction procedure, demonstrate the effectiveness of the present quantitative method. It is also interesting to note that our values for dehydroisoandrosterone are somewhat higher than those reported by other investigators using methods which employ various hydrolytic procedures.

In the limited number of subjects studied thus far, 17-ketosteroids were recovered solely as sulfates. The presence of 1 to 2 μg of androsterone per 100 ml of plasma after enzyme hydrolysis (glucuronidase) has been reported by others (3, 21). Since androsterone glucuronide added to the 15-ml samples of plasma has been adequately recovered, it is evident that glucuronides can be detected by the present method, but the small amounts of endogenous androsterone glucuronide (e.g., < 0.5 μg per 100 ml) present in samples of such size are not detectable. Minimal amounts also may be circulating as free alcohols as proposed by Cohn, Bondy and Castiglione (22), or perhaps as esters of other acids. The lower limit of sensitivity of the present method (15 ml of plasma) is 15 μg of steroid conjugates per 100 ml; any smaller amount would not be detected. However, it is apparent that the ratio of sulfates to glucuronides found in urine (16) is not present in plasma. This suggests either a more rapid clearance of glucuronides than of sulfates (21), or a renal conjugating mechanism by which glucuronic acid displaces the sulfate radical. The formation of glucuronides of free steroids in the kidney (23) is another possibility.

The direct quantification of the Zimmermann material on paper deserves further comment. This was first proposed by Oertel (24) and Bush and Willoughby (25), using a slightly different apparatus. The main advantage of such a method is that all problems encountered during elution of compounds are avoided. Some of the problems frequently encountered are: introduction of impurities from paper and solvents, loss of material, or the overlooking of "unexpected" spots when areas adjacent to reference standards are cut out without staining pilot strips. (Omission of pilot strips may become necessary when working with very small amounts of steroids.) Also, inadequate separation of compounds with closely related Rf values may lead to over- or underestimation through arbitrary selection of the areas to be eluted. Finally, the solvents used in the present system of paper chromatography seemed to interfere seriously with the Zimmermann reaction when quantification was attempted after elution, making this technique impractical, if not impossible.

Direct quantification through planimetric scanning procedures, as practiced here, also has its disadvantages, one of which is the rigorous time schedule which must be observed while staining and reading the strips. The greatest drawback, however, is its limited reproducibility, particularly when tailing or spreading of spots is excessive. This can be limited by careful application of the samples to paper and, as shown above, by triplicate readings. In our opinion, then, direct quantification should be given serious consideration, particularly when paper chromatography is part of a clinical method.

SUMMARY

A method for the direct estimation of dehydroisoandrosterone sulfate and androsterone sulfate in plasma has been presented. The procedure includes extraction, column and paper chromatography, and direct quantification on paper by planimetric evaluation. It has been shown that the criteria for recovery reproducibility and specificity of this method have been fulfilled. Plasma samples from normal men and women of reproductive age have been studied, as well as a limited number of samples obtained from female patients with various virilizing disorders. The normal values seem to be somewhat higher than those obtained by other investigators with a method involving hydrolytic procedures. This, we believe, can be explained by the greater molecular weight of the steroid esters as compared with the free alcohol, the greater Zimmermann chromogenicity of androsterone sulfate as compared with androsterone on a molar basis, and a greater recovery, since the loss occurring during hydrolysis was avoided. Free steroids or glucuronides of steroids were not detected with the present method. The reason for this has been discussed.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Rezan Berki, Mr. John Hageman and Miss Fritze Pedersen for their assistance.

REFERENCES

1. Finkelstein, M., Forchielli, E., and Dorfman, R. I. The estimation of testosterone in human plasma. J. clin. Endocr. 1961, **21**, 98.
2. Butt, W. R., and Crooke, A. C. The disappearance rate of intravenously injected testosterone and progesterone from the human circulation (abstract). J. Endocr. 1950, **7**, xxviii.
3. Clayton, G. W., Bongiovanni, A. M., and Papadatos, C. Preliminary investigations into the nature of neutral 17-ketosteroids in human plasma. J. clin. Endocr. 1955, **15**, 693.
4. Lombardo, M. E., Mann, P. H., Viscelli, T. A., and Hudson, P. B. A method for the extraction of steroids from blood. J. biol. Chem. 1955, **212**, 345.
5. Migeon, C. J., and Plager, J. E. A method for the fractionation and measurement of 17-ketosteroids in human plasma. J. clin. Endocr. 1955, **15**, 702.
6. Oertel, G. W., and Eik-Nes, K. Determination of dehydroepiandrosterone in human blood plasma. J. biol. Chem. 1958, **232**, 543.
7. Tamm, J., Beckmann, I., and Voigt, K. D. Determination of neutral steroids in human blood. Acta Endocr. 1957, suppl. **31**, 219.
8. West, C. D., Tyler, F. H., Brown, H., and Samuels, L. T. The role of the liver and kidneys in the metabolism of intravenous testosterone by human subjects. J. clin. Endocr. 1951, **11**, 897.
9. Ceresa, F., and Cravetto, C. A. A method for the simultaneous assay of 17-hydroxycorticosteroids and 17-ketosteroids both in their free and conjugated forms on the same plasma sample. Acta Endocr (Kbh.) 1958, **29**, 321.
10. Dumazert, C., and Valensi, G. Sur le dosage des 17-cétostéroïdes neutres du sang. C. R. Soc. Biol. (Paris) 1952, **146**, 471.
11. Saier, E. L., Campbell, E., Strickler, H. S., and Grauer, R. C. A simplified method for the simultaneous determination of 17-ketosteroids, dehydroepiandrosterone, and 17-hydroxycorticosteroids in serum. J. clin. Endocr. 1959, **19**, 1162.
12. Simmer, H., Simmer, I. and Zellmer, O. Androgene im peripheren Veneblut von gesunden und an spätgestosen erkrankten Schwangeren. Klin. Wschr. 1959, **37**, 966.
13. Baulieu, E. E. Three sulfate esters of 17-ketosteroids in the plasma of normal subjects and after administration of ACTH. J. clin. Endocr. 1960, **20**, 900.
14. Herrmann, W., and Silverman, L. Method for determination of urinary pregnane-3-alpha, 17-alpha, 20-alpha triol. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 426.
15. Bush, I. E. Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J. 1952, **50**, 370.
16. Schneider, J. J., and Lewbart, M. L. Fractionation and isolation of steroid conjugates. Recent Progr. Hormone Res. 1959, **15**, 201.
17. Bush, I. E. Quantitative estimations of steroids by direct scanning of paper chromatograms *in* Memoir of the Society for Endocrinology (no. 8). Quantitative Paper Chromatography of Steroids, D. Abelson and R. V. Brooks, Eds. London, Cambridge Univ. Press, 1960, p. 24.
18. Bush, I. E. The possibilities and limitations of paper chromatography as a method of steroid analysis. Recent Progr. Hormone Res. 1954, **9**, 321.
19. Oppenauer, R. V. Dehydration of secondary alcohols to ketones. I. Preparation of sterol ketones and sex hormones. Rec. Trav. chim. Pays-Bas. 1937, **56**, 137.
20. Schneider, J. J., and Lewbart, M. L. Enzymatic synthesis of steroid sulfates. J. biol. Chem. 1956, **222**, 787.
21. Kellie, A. E., and Smith, E. R. Renal clearance of 17-oxo steroid conjugates found in human peripheral plasma. Biochem. J. 1957, **66**, 490.
22. Cohn, G. L., Bondy, P. K., and Castiglione, C. Studies on pyrogenic steroids. I. Separation, identification, and measurement of unconjugated dehydroepiandrosterone, etiocholanolone, and androsterone in human plasma. J. clin. Invest. 1961, **40**, 400.
23. Cohn, G. L., and Hume, M. The *in vivo* glucuronide conjugation of radioactive etiocholanolone and androsterone by the dog kidney. J. clin. Invest. 1960, **39**, 1584.
24. Oertel, G. Papierchromatographische Trennung und Bestimmung von 17-Ketosteroiden. Acta Endocr. (Kbh.) 1954, **16**, 263.
25. Bush, I. E., and Willoughby, M. The excretion of *allo*-tetrahydrocortisol in human urine. Biochem. J. 1957, **67**, 689.

HEARTBURN IN LATE PREGNANCY. MANOMETRIC STUDIES OF ESOPHAGEAL MOTOR FUNCTION *

By RICHARD NAGLER and HOWARD M. SPIRO

(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.)

(Submitted for publication May 27, 1960; accepted January 13, 1961)

Heartburn is a burning, often hot sensation which may localize in the throat or substernal region. It is especially common during pregnancy. Gastroesophageal reflux and neuromuscular dysfunction of the esophagus have been suggested as underlying pathophysiological mechanisms, but contradictory evidence has prevented a clear definition of the cause of heartburn.

A prospective clinical study of 560 pregnant women disclosed that 50 per cent developed heartburn which invariably disappeared after delivery (1). The self-limited character of pregnancy makes it an ideal experimental period during which to study heartburn. Transducer pressure recordings of esophageal motility are well adapted to such a study.

This paper reports the results of serial esophageal motility studies in pregnant women with and without heartburn and in a group of asymptomatic normal women of similar age.

METHODS

Subjects. Fifty-seven esophageal motility studies were performed on 20 pregnant women who complained of heartburn during pregnancy. The 8 primiparae and 12 multiparae ranged in age from 16 to 39 years, with an average age of 28. Similarly, 63 recordings were obtained from 19 pregnant women who never developed heartburn throughout pregnancy; in this group were 10 primiparae and 9 multiparae, 20 to 35 years old, with an average age of 26. Each women was studied at least once during and after pregnancy, but the total number of studies in each subject depended on the month of pregnancy when the patient was first seen. Fourteen asymptomatic normal nonpregnant women, 22 to 44 years old, with an average age of 26, served as controls; each subject was studied 6 times at monthly intervals.

Technique. The technique of recording esophageal motility utilizes 3 polyethylene catheters, each with an internal diameter of 1.5 mm, which are fastened together and sealed at the tips. A single lateral opening in each at 5-cm intervals permits simultaneous recording of pressure changes developing from 3 sites within the esophagus and its sphincters. The catheters are water-filled and transmit pressures to 3 Sanborn electromanometers, the changes in pressure being recorded on a Sanborn multi-channel direct writing recorder. During every recording the vertical position of the electromanometers is readjusted to the same level as the subject's esophagus. This minimizes any hydrostatic effect and permits consistency in serial recording techniques. Swallowing is signaled by simultaneous recordings of laryngeal movement by means of a specially adapted Sanborn pulse wave attachment placed on the skin adjacent to the thyroid cartilage. A pneumographic belt around the chest indicates respiratory movements.

Patients fast for a minimum of 6 hours prior to examination. The catheters are passed through the mouth into the stomach without the use of a local anesthetic. With the patient supine, they are withdrawn, centimeter by centimeter, while pressure changes are continuously recorded in the resting state, and also following a "dry" swallow and the ingestion of approximately 10 ml of water.

When the tip of the catheter lies below the diaphragm, inspiration produces a positive pressure response which reflects increased intra-abdominal pressure. When the tip is above the diaphragm, the normal decrease in pressure in the thorax on inspiration produces a negative deflection. This point of pressure reversal occurs at the diaphragm and is called the "effective diaphragmatic hiatus." This convenient physiological landmark permits localization of the catheters without the use of X-ray or fluoroscopy.

All records are analyzed in a uniform manner. The zero point is the pressure recorded from the stomach 5 cm distal to the point of respiratory reversal in each subject. The catheters are held in this position, as much as possible, by a trained technician to avoid any vertical movement. The fundal esophageal pressure gradient is obtained by subtracting the mean resting intra-esophageal pressure from the mean resting fundal or zero pressure. A high pressure zone from 1 to 4 cm long, extending usually from just below the effective diaphragmatic hiatus to a point 1 to 2 cm above it, functions as an intrinsic sphincter mechanism and is called the inferior esophageal sphincter or vestibule (2–5). Sphincter tone is recorded as the mean resting end-expiratory pressure, which is represented by the pressure gradient between the fundus of the stomach and the sphincter during expiration.

* Supported in part by United States Public Health Service Grants C2578, A1785, and A3473 and the Norwich Chapter of the Connecticut Heart Association.

The best defined resting pressure recorded during withdrawal of the 3 catheters through the sphincter was selected as representative. Relative to resting sphincter pressure, negative and positive readings in this zone develop in response to deglutition and are believed to represent relaxation and contraction of the sphincter, respectively; these are expressed only in qualitative terms. Primary peristalsis (Figure 2A) is represented by the peak positive pressures produced by the muscular contraction moving down the esophagus in response to swallowing (6–10). Both maximal and mean pressures from the middle third of the esophagus are tabulated. Secondary peristalsis (Figure 1) differs from primary peristalsis only in the mode of initiation; primary peristaltic waves follow deglutition while secondary peristaltic waves are initiated by distention (11–13). The frequency of nonpropulsive responses to swallowing (Figure 2B, C) is expressed as a percentage of the total number of swallows. An effort was made to standardize the number of swallows per recording, but a few individuals were less cooperative and swallowed more often. We have arbitrarily considered nonpropulsive responses to more than 25 per cent of the swallows as abnormal. This is based upon the observation that almost all of our asymptomatic, young, normal control subjects had less than this percentage of nonpropulsive responses to swallowing. Spontaneous nonpropulsive contractions (Figure 2D) are designated as "only a few" (less than 10

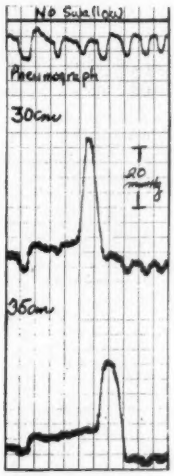


FIG. 1. ELECTROMANOMETRIC RECORDING OF A SECONDARY PERISTALTIC WAVE. This secondary peristaltic wave is propulsive but not preceded by a swallow, which is the only differentiating feature from the primary peristaltic wave that follows swallowing (Figure 2A). The numbers on the left are distances from the incisors. The recording speed used in Figures 1 through 8 was 2.5 mm per second.

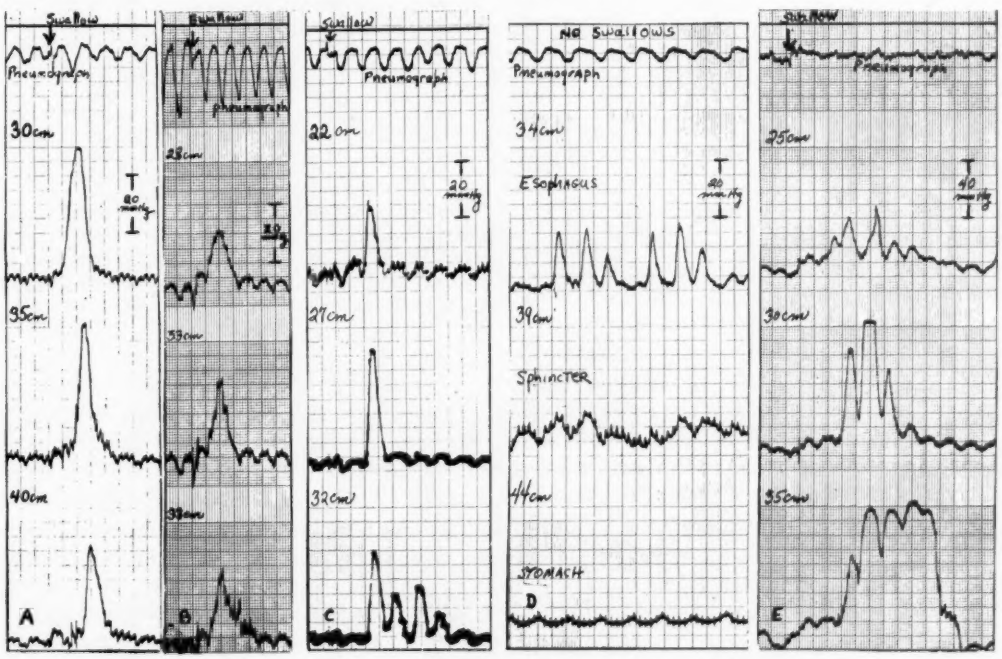


FIG. 2. TYPICAL ESOPHAGEAL RESPONSES OF VARIOUS PREGNANT WOMEN. A) Normal propulsive response to a "dry" swallow; B) nonpropulsive response to a "dry" swallow; C) nonpropulsive repetitive response to a "dry" swallow; D) nonpropulsive repetitive response not preceded by a swallow; E) diffuse esophageal spasm after a "dry" swallow. The numbers on the left of each panel are distances from the incisors.

TABLE I

Control subjects *

Subject	Age	Study no.	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph.		Nonprop. cont.	
					Max.	Mean	% Total swallows	Not prec. by swallow
	yrs		*mm Hg*		*mm Hg*			
P.C.	23	1	4	16	55	40	<5	0
		2	4	15	45	35	<5	Few
		3	5	12	45	25	0	0
		4	4	15	40	25	0	Few
		5	4	15	50	25	<5	Few
		6	5	12	50	30	0	0
A.M.	24	1	5	12	50	35	25	0
		2	6	10	45	35	33	0
		3	5	10	40	20	20	0
		4	5	14	50	35	0	0
		5	5	10	40	25	0	0
		6	5	10	45	30	20	0
M.H.	22	1	3	9	45	35	0	0
		2	2	8	45	30	<5	0
		3	3	9	40	30	0	0
		4	2	10	45	30	<5	0
		5	3	8	45	25	10	0
		6	2	10	40	25	0	0
L.H.	34	1	2	4	90	50	<5	0
		2	2	6	70	30	<5	0
		3	2	5	60	45	0	0
		4	2	5	60	30	0	0
		5	3	5	50	35	0	0
		6	2	6	55	40	0	0
D.G.	44	1	3	14	60	40	33	Many
		2	3	12	50	35	10	Few
		3	3	12	50	35	50	Few
		4	4	13	50	40	33	Many
		5	4	10	45	30	0	Few
		6	4	12	50	25	20	Few
E.L.	23	1	4	9	45	30	5	0
		2	4	10	35	25	5	0
		3	5	14	45	25	<5	0
		4	4	9	35	25	0	0
		5	4	12	35	20	0	0
		6	5	10	35	20	5	0
H.N.	22	1	4	10	55	40	0	0
		2	3	9	50	40	<5	0
		5	3	9	60	45	0	0
		4	3	11	70	40	<5	0
		5	4	9	65	45	0	0
		6	3	10	50	35	0	0
A.M.	27	1	6	5	50	35	40	0
		2	7	8	50	35	67	Few
		3	6	5	55	35	67	Many
		4	7	8	55	35	33	Few
		5	7	7	45	30	40	Few
		6	7	8	45	25	5	0
F.M.	27	1	2	1	45	25	0	0
		2	2	3	45	20	0	0
		3	2	2	45	25	60	0
		4	3	1	45	30	0	0
		5	3	2	45	30	40	0
		6	2	2	40	20	0	0

* No diffuse spasm or secondary peristalsis occurred in any of the control subjects.

TABLE I—(*Continued*)

Subject	Age	Study no.	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph.		Nonprop. cont.	
					Max.	Mean	% Total swallows	Not prec. by swallow
	yrs		*mm Hg*		*mm Hg*			
B.V.	22	1	4	11	40	30	10	0
		2	5	11	45	25	0	Few
		3	4	10	50	30	10	0
		4	4	8	40	30	<5	Few
		5	5	9	35	25	<5	Few
		6	5	12	40	25	0	0
H.T.	23	1	3	5	60	40	<5	0
		2	4	6	50	35	0	Few
		3	3	6	70	40	0	0
		4	3	5	50	35	20	0
		5	4	7	45	30	20	Few
		6	3	5	40	25	30	0
C.P.	23	1	3	7	35	25	5	0
		2	2	8	45	25	0	0
		3	3	8	40	25	0	0
		4	3	10	40	30	<5	0
		5	2	10	45	30	0	0
		6	2	9	45	25	0	0
J.D.	23	1	3	9	50	30	50	Many
		2	4	12	55	30	10	Few
		3	4	10	60	30	<5	0
		4	4	10	50	25	50	Many
		5	3	10	50	35	30	Few
		6	4	14	55	30	0	Few
J.W.	24	1	3	2	25	15	0	0
		2	2	2	25	15	<5	0
		3	3	3	25	15	0	0
		4	3	2	25	15	0	0
		5	3	3	20	15	0	0
		6	3	2	25	15	0	0

per recording) or "many" (greater than 10 per recording). Each recording extended for approximately 20 minutes. The term "diffuse spasm" (Figure 2E) specifically describes forceful, prolonged, repetitive, nonpropulsive contractions in the lower two-thirds of the esophagus (14).

RESULTS

Control subjects. Although the pressure profile varies among individuals (Table I), certain findings are characteristic of the control group. Diffuse esophageal spasm and secondary peristalsis were not seen. The average fundal-esophageal pressure gradient of 3 to 4 mm of mercury was constant on serial studies on each subject. The inferior esophageal sphincter (represented by the difference of pressure between the high pressure zone and the gastric fundus) measured from 1 to 16 mm of mercury. In contrast to the observed variation from one individual to another, serial studies of the sphincter on the same subject generally demonstrated consistently reproducible pressure profiles. The consistency of this pressure response was notable. After a "dry" swallow, a fall in pressure followed by a rise above resting pressure took place in the sphincter region of all control subjects.

Primary peristaltic pressures from 15 to 90 mm of mercury delineated the normal range. On serial study, 6 subjects inconsistently developed frequent nonpropulsive responses to "dry" swallowing, although a normal peristaltic response invariably followed a swallow of 10 ml of water. Numerous simultaneous contractions not preceded by a swallow occurred in only 3 patients.

Pregnant women without heartburn. As in the control subjects, variability exists among individuals (Table II). Since these women are subject to the physiological effects of an advancing

TABLE II

Pregnant women without heartburn *

Patient	Age	Month of pregnancy studied	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph.		Nonprop. cont.	
					Max.	Mean	% Total swallows	Not prec. by swallow
	yrs		*mm Hg*		*mm Hg*			
D.R.	32P	6	2	10	80	40	40	0
		8	3	6	100	45	50	Few
		PP	3	10	70	35	5	0
S.M.	24P	4	5	10	55	45	25	0
		5	6	12	50	40	20	0
		6	7	12	50	40	50	Many
		7	8	10	55	45	40	Few
		8	9	10	45	35	30	Few
		PP	3	10	50	40	<5	0
P.S.	26P	5	2	9	50	30	20	0
		9	6	10	40	20	50	0
		PP	3	8	50	30	0	0
S.K.	20M	7	2	10	50	25	30	0
		8	9	10	40	25	30	0
		PP	4	9	45	25	50	0
E.W.	23M	4	6	7	60	30	50	0
		5	8	6	50	30	70	Many
		6	8	7	70	40	40	Few
		7	7	8	55	30	70	Few
		8	8	7	50	30	70	Many
		PP	5	7	50	30	50	Many
J.J.	28M	5	8	9	55	30	5	0
		8	10	10	50	35	0	0
		PP	6	12	50	35	0	0
S.F.	23P	4	5	6	40	30	50	0
		5	4	4	45	20	70	Many
		6	4	5	30	20	30	Few
		7	4	6	30	20	66	Many
		9	5	6	40	25	50	Many
		PP	4	5	35	20	<5	Few
S.B.	21P	6	8	12	50	30	80	Many
		7	9	10	55	40	40	Few
		9	8	10	50	30	33	0
		PP	6	10	50	35	0	0
N.S.	22M	8	4	2	25	15	90	Few
		PP	3	3	35	20	60	Few
C.S.	26P	7	5	6	50	35	70	Few
		PP	5	10	55	35	25	Many
A.E.	26M	8	5	5	50	40	30	Few
		PP	2	5	50	30	0	Few
E.V.	35M	6	3	7	25	15	70	Few
		8	7	3	20	15	30	0
		PP	2	10	25	15	<5	0
M.S.	27M	8	5	1	45	25	40	0
		9	5	2	40	20	50	Few
		PP	2	5	40	20	<5	0
I.G.	35M	8	4	8	45	35	10	0
		PP	4	9	50	30	0	0

* P = primipara; M = multipara; PP = post partum. No diffuse spasm or secondary peristalsis occurred in the pregnant women without heartburn.

TABLE II—(*Continued*)

Patient	Age	Month of pregnancy studied	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph. Max.	Peristaltic press. Middle ⅓ esoph. Mean	Nonprop. cont. % Total swallows	Nonprop. cont. Not prec. by swallow
	yrs		*mm Hg*		*mm Hg*			
L.W.	26P	6	2	5	30	15	60	Few
		8	4	3	35	20	25	Few
		9	6	2	35	15	30	0
		PP	2	6	35	15	<5	0
D.G.	23P	6	5	5	45	30	80	Few
		8	8	6	50	30	60	Few
		PP	3	5	40	25	60	Few
F.D.	25P	8	5	6	35	10	60	0
		PP	3	5	35	25	0	0
N.H.	24M	9	7	6	40	25	40	Many
		PP	3	7	45	20	60	Few
C.C.	31P	6	5	10	55	35	50	O
		8	8	12	65	40	30	0
		PP	5	10	80	40	40	Few

pregnancy, the results of serial esophageal motility changes during pregnancy are presented, each woman serving as her own control. Neither diffuse esophageal spasm nor secondary peristalsis was seen. The average fundal esophageal pressure gradient of 6 to 7 mm of mercury was higher than that recorded in the controls. This gradient generally rose with advancing pregnancy and fell post partum to levels similar to those determined in the normal nonpregnant group. The mean resting end-expiratory pressure developed by the inferior esophageal sphincter varied from 1 to 12 mm of mercury. Fourteen women maintained a consistent sphincter pressure profile throughout pregnancy and after delivery, while five subjects demonstrated a fall in sphincteric pressure as pregnancy advanced, with a return to previous levels post partum. After a "dry" swallow, a fall in sphincter pressure followed by a rise above resting levels occurred in all but two patients during pregnancy. Both of these women demonstrated a barely perceptible high pressure zone. The return of normal sphincteric function after delivery coincided with a rise in the resting sphincter pressure. During pregnancy, the inferior esophageal sphincter was confined at or just above the effective diaphragmatic hiatus in 63 per cent of subjects. This represented a significant departure from its usual anatomical position, which in most women ordinarily included an intra-abdominal segment.

Primary peristaltic pressures ranged from 10 to 100 mm of mercury and compared closely with control values. Seventeen women consistently developed frequent nonpropulsive pressure responses to "dry" swallowing during pregnancy, although a normal peristaltic wave usually followed a swallow of 10 ml of water. Six women showed numerous nonpropulsive contractions not preceded by a swallow. After delivery, the incidence of abnormal motor responses approached the controls. Neither parity nor age correlated with the changes observed.

Pregnant women with heartburn. The results (Table III) illustrate the changing esophageal pressure profile during pregnancy, each woman serving as her own control. Forty-five per cent demonstrated secondary peristalsis which disappeared post partum, but which was never seen in either of the other two groups. The average fundal esophageal pressure gradient of 6 to 7 mm of mercury was similar to that of the group of pregnant women without heartburn, but higher than that of the controls. This gradient generally rose with advancing pregnancy and fell post partum to levels similar to those found in the normal nonpregnant group. The mean resting end-expiratory pressure of the inferior esophageal

TABLE III

Pregnant women with heartburn *

Patient	Age	Month of pregnancy studied	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph.		Nonprop. cont.		Diffuse spasm	Secondary peristalsis
					Max.	Mean	% Total swallows	Not prec. by swallow		
	yrs		*mm Hg*		*mm Hg*					
J.W.	21P	7	3	5	70	50	<5	0		+
		9	5	3	45	25	0	0		+
		PP	2	8	45	30	0	0		
D.B.	28M	9	8	1	55	40	<5	0		
		PP	4	6	45	35	0	0		
R.T.	29M	9	3	7	90	45	90	Many	+	
		PP	4	8	140	70	90	Few	+	
A.D.	30P	6	5	9	45	35	<5	0		
		7	7	6	35	25	10	0		
		8	8	3	45	30	80	Few		+
		PP	3	10	45	25	0	0		
R.K.	31M	8	5	5	50	25	5	0		
		9	7	6	40	25	10	0		
		PP	3	6	40	20	<5	0		
B.H.	25P	8	8	8	60	40	75	Few	+	
		PP	4	7	70	40	70	Many	+	
B.R.	25M	6	5	12	50	35	20	Many		
		8	9	6	50	30	50	Few		
		PP	4	10	70	45	30	Few		
H.U.	39M	8	6	4	30	15	50	0		
		9	8	5	30	25	70	0		
		PP	5	5	30	20	5	0		
J.H.	29P	9	4	6	80	35	75	Many	+	
		PP	3	5	95	40	50	Many	+	
M.R.	25M	4	5	5	20	15	75	Few		
		6	6	4	30	15	60	Few		
		7	8	3	30	10	90	Many		+
		8	9	2	25	10	60	Many		
		9	8	2	20	10	90	Many		+
		PP	5	6	20	10	<5	Few		
R.C.	35M	8	7	12	100	50	67	0		
		9	6	15	110	50	67	0		
		PP	2	12	80	35	33	0		
P.M.	21P	9	8	2	40	30	<5	0		+
		PP	3	6	50	40	<5	0		
J.L.	27M	8	7	2	60	40	50	Few		+
		9	7	1	50	35	50	Few		+
		PP	2	4	50	35	0	0		
C.F.	26M	7	4	6	45	35	30	Few		
		8	5	5	50	35	50	Few		
		PP	5	9	45	30	5	0		
E.H.	35M	9	9	0	40	20	0	0		+
		PP	3	6	45	25	0	0		
D.W.	23P	9	8	2	25	15	60	Few		+
		PP	5	3	40	20	50	0		

* P = primipara; M = multipara; PP = post partum.

TABLE III—(*Continued*)

Patient	Age	Month of pregnancy studied	Fundal esoph. press. grad.	Inf. esoph. sph. Mean rest. end-exp. press.	Peristaltic press. Middle ⅓ esoph. Max.	Peristaltic press. Middle ⅓ esoph. Mean	Nonprop. cont. % Total swallows	Nonprop. cont. Not prec. by swallow	Diffuse spasm	Secondary peristalsis
	yrs		*mm Hg*		*mm Hg*					
H.C.	38M	9	5	5	45	30	80	Many		+
		PP	2	5	40	25	20	0		
E.M.	16P	9	5	2	45	25	60	Few		
		PP	4	6	55	30	0	Few		
A.V.	18P	7	6	8	55	30	50	Few		
		9	7	4	45	20	75	Few		
		PP	4	10	45	25	50	Few		
E.D.	30M	5	3	6	40	25	50	0		
		6	5	3	50	25	33	Few		
		7	6	3	50	30	75	Many		+
		9	8	2	45	20	50	Many		+
		PP	2	8	40	25	<5	Few		

sphincter varied from 0 to 15 mm of mercury. Eight women maintained a consistent sphincter pressure profile during pregnancy and post partum while 12 demonstrated a fall in sphincteric pressure which returned to previous levels after delivery. The response to a "dry" swallow was present in all but two patients during pregnancy. In one woman the inferior esophageal sphincter

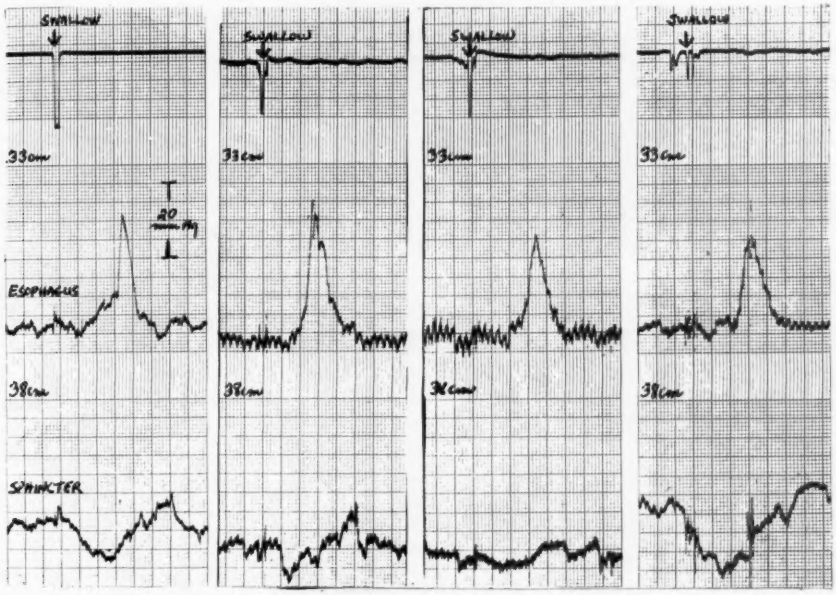


FIG. 3. ELECTROMANOMETRIC RECORDINGS OF PATIENT E. D. IN THE FIFTH, SEVENTH AND EIGHTH MONTHS OF PREGNANCY, AND AFTER DELIVERY (LEFT TO RIGHT). Note the progressive diminution of inferior esophageal sphincter response to a "dry" swallow with advancing pregnancy and the return to normal post partum. The patient experienced heartburn frequently in the last trimester of pregnancy. Calibrations are the same throughout. The numbers on the left of each panel are distances from the incisors.

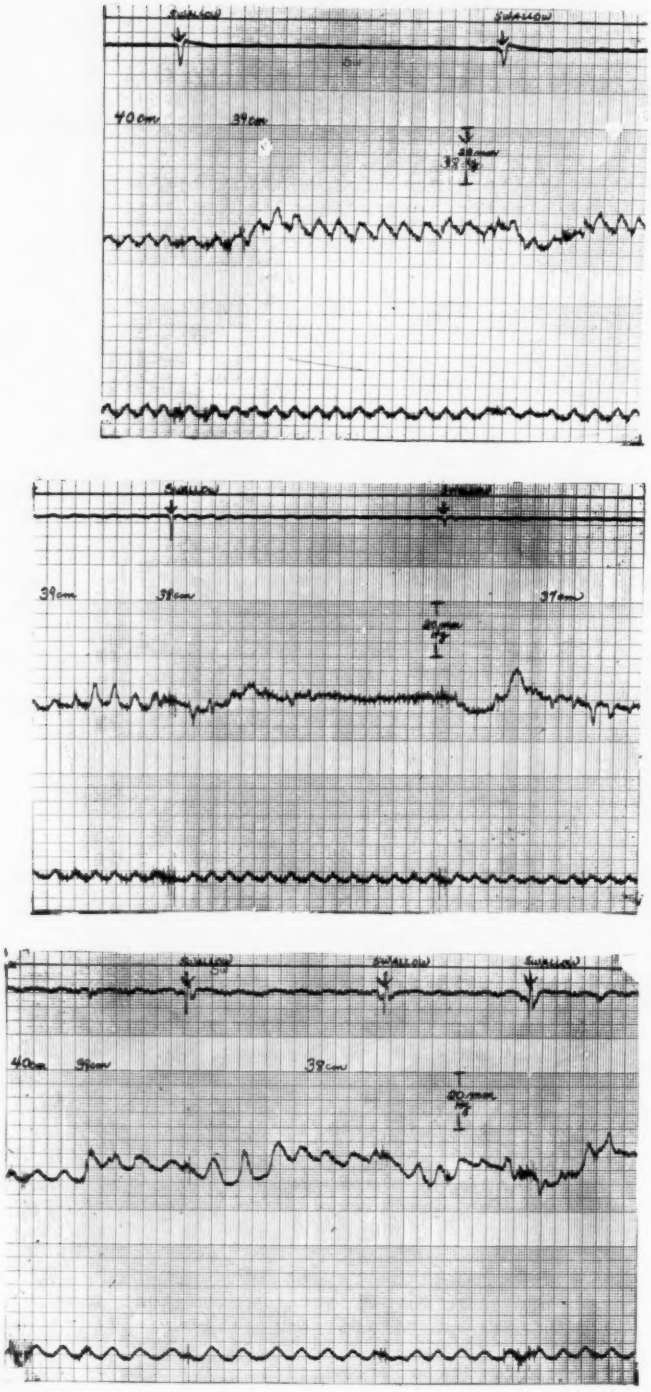


FIG. 4. ELECTROMANOMETRIC RECORDINGS OF PATIENT M. R. IN THE FOURTH (TOP PANEL) AND SEVENTH (MIDDLE PANEL) MONTHS OF PREGNANCY, AND AFTER DELIVERY (BOTTOM PANEL). The numbers across the top of each panel are distances from the incisors. The middle tracing on each panel is from the sphincter; the bottom tracing is from the fundus. Top panel: Respiratory responses in the fundus and sphincter are the same indicating that the sphincter is below the effective diaphragmatic hiatus, i.e., an intra-abdominal segment first identified 39 cm from the incisors. Middle panel: Respiratory responses in the fundus and sphincter are opposite indicating that the entire sphincter is in the thorax beginning 38 cm from the incisors at the point of respiratory reversal. Bottom panel: The intra-abdominal segment of the sphincter reappears 39 cm from the incisors. Several drops in pressure following a swallow are evident.

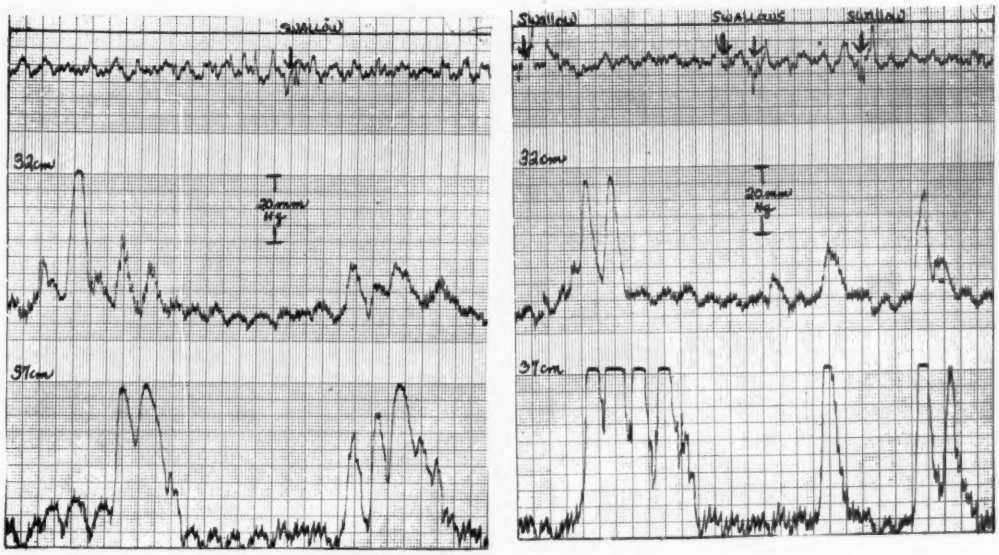


FIG. 5. ELECTROMANOMETRIC RECORDINGS OF PATIENT B. H. IN THE EIGHTH MONTH OF PREGNANCY (LEFT) AND 2 MONTHS AFTER DELIVERY (RIGHT). The numbers at the left of each panel are distances from the incisors. Left panel: Diffuse esophageal spasm. The patient had severe heartburn during the third trimester of pregnancy. Right panel: Diffuse esophageal spasm still present. The patient had been completely asymptomatic since delivery.

could not be demonstrated, and in the other it was barely perceptible (Figure 3). Both patients, however, revealed normal function post partum, coincident with a substantial rise in the resting sphincteric pressure. A most important finding during pregnancy was that an intra-abdominal segment of the sphincter was identified in only 15 per cent of women in this group; it reappeared after delivery in most subjects (Figure 4).

Three women had a pressure profile typical of diffuse esophageal spasm, following both a "dry" and "wet" swallow, but in the asymptomatic postpartum period, motility remained similarly disturbed (Figure 5). In each instance, the inferior esophageal sphincter was well developed and demonstrated normal function.

Primary peristaltic pressures varied from 10 to 110 mm of mercury, which compared closely with control values. Fifteen women consistently developed frequent nonpropulsive pressure responses to "dry" swallowing during pregnancy, although a normal primary peristaltic wave usually followed a swallow of 10 ml of water. Seven women showed numerous simultaneous contractions not preceded by a swallow. After delivery, the frequency of abnormal motor responses approached control values. Neither parity nor age correlated with these results.

Three women experienced heartburn during the manometric study and are reported in greater detail.

Subject 1. J. L., a 27 year old gravida II, had heartburn for the first time in the sixth month of pregnancy. Episodes of a burning, hot sensation localized to the throat, associated with the occurrence of sour-tasting fluid in the mouth, were brought on by large meals and the supine position. Antacids gave prompt relief. During an esophageal motility study performed in the eighth month of pregnancy, the patient complained of waves of heartburn; simultaneously, secondary peristaltic waves appeared on the graph (Figure 6). The fundal esophageal pressure gradient was 7 mm of mercury. The inferior esophageal sphincter was located entirely above the effective diaphragmatic hiatus and developed a mean resting end-expiratory pressure of 2 mm of mercury. Numerous nonpropulsive pressures developed both spontaneously and in response to swallowing. During the ninth month of pregnancy, heartburn again was associated with the appearance of secondary peristaltic waves. With the catheters in place, the pa-

tient was given 30 ml of a liquid antacid which promptly relieved the heartburn. The motility record, however, was unchanged.

After delivery the patient became asymptomatic, and a recording 6 weeks later was normal. The fundal esophageal pressure gradient fell to 2 mm of mercury and the inferior esophageal sphincter which was now observed, both below and above the effective diaphragmatic hiatus, developed a resting pressure of 4 mm of mercury.

Comment. Esophageal motility studies demonstrated secondary peristalsis simultaneously with symptoms of heartburn. Nonpropulsive activity was also present. A liquid antacid effectively relieved the heartburn, but the pressure profile was unchanged, suggesting that motor phenomena are not the only determinants of heartburn.

Subject 2. E. H., a 35 year old gravida II, had heartburn for the first time in the seventh month of pregnancy. A burning sensation in the substernal area radiating to the throat was precipitated by bending, stooping, or lying flat especially after eating. All antacid preparations nauseated her. The patient complained of persistent heartburn during an esophageal motility study performed in the ninth month of pregnancy, and again secondary peristalsis was seen (Figure 7). The fundal esophageal pressure gradient was 9 mm of mercury, the inferior esophageal sphincter could not be identified, and nonpropulsive activity was not observed.

The pressure profile was normal 8 weeks post partum; she had experienced no heartburn after delivery. The fundal esophageal pressure gradient fell to 3 mm of mercury and the inferior esophageal sphincter could now be identified both below and above the effective diaphragmatic hiatus with a mean resting end-expiratory pressure of 6 mm of mercury. A pneumatic bag was then placed around the patient's waist and 300 ml of water rapidly instilled directly into the stomach. Upon inflation of the bag to exert a pressure of 100 mm

FIG. 6. ELECTROMANOMETRIC RECORDINGS OF SUBJECT 1 (J. L.). The numbers at the left are distances from the incisors. A and D are secondary peristaltic responses not preceded by a swallow. B and C are primary peristaltic waves after a swallow as the patient attempted to clear acid-tasting material which had refluxed into the throat.

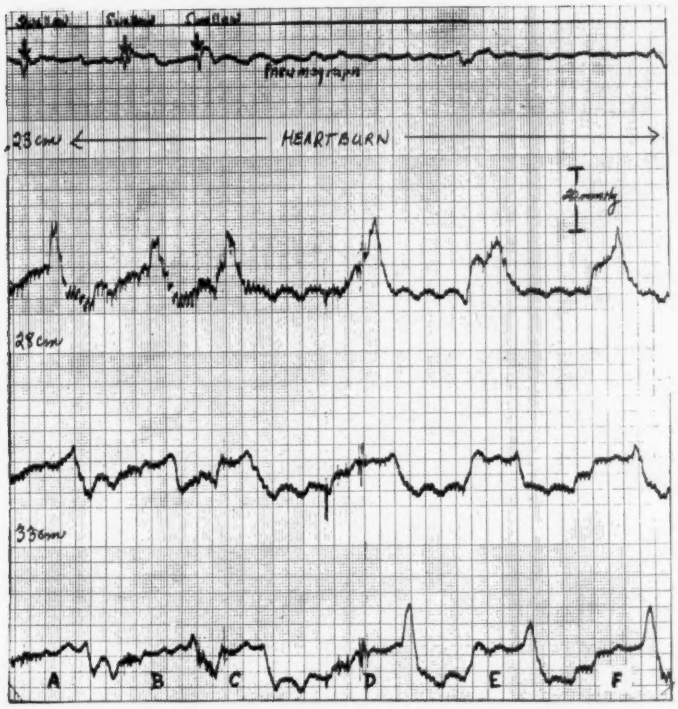


FIG. 7. ELECTROMANOMETRIC RECORDINGS OF SUBJECT 2 (E. H.). The numbers at the left are distances from the incisors. A, B and C are primary peristaltic responses after a swallow. D, E and F are secondary peristaltic waves not preceded by a swallow. The patient had heartburn throughout this recording.

of mercury secondary peristalsis promptly ensued, but she experienced no heartburn.

Comment. Heartburn occurred during the manometric demonstration of secondary peristalsis in the absence of nonpropulsive motor activity. A similar pressure profile induced post partum was observed without heartburn, suggesting that heartburn does not depend on secondary peristalsis.

Subject 3. H. C., a 38 year old gravida V, had heartburn for the first time in the sixth month of pregnancy. A burning, choking sensation in the throat associated with the occurrence of sour-tasting material in the mouth was aggravated by emotional tension and the supine position especially after meals. She never tried antacids, but noted transient relief from drinking a glass of cold water. An esophageal motility study in the ninth month of pregnancy demonstrated secondary peristalsis and prominent nonpropulsive activity not preceded by a swallow. During the first part of the study she complained of heartburn. Immediately after a single swallow of approximately 20 ml of water at room temperature, a normal primary peristaltic wave developed which was promptly followed simultaneously by the sudden cessation of heartburn and an abrupt change in the motility pattern to normal (Figure 8). The fundal esophageal pressure gradient was 5 mm of mercury. Inferior esophageal sphincter activity occurred only above the effective diaphragmatic hiatus and developed a mean resting end-expiratory pressure of 5 mm of mercury.

She became asymptomatic after delivery; a follow-up motility study 7 weeks later was normal. The fundal esophageal pressure gradient fell to 2 mm of mercury, but the mean resting end-expiratory pressure of the inferior esophageal sphincter, however, could now be identified. A pneumatic belt was then placed about her waist and 300 ml of water instilled directly into the stomach. Upon

inflation of the bag to 100 mm of mercury, secondary peristalsis and spontaneous nonpropulsive waves promptly occurred, but the patient experienced no heartburn.

Comment. Heartburn was associated with secondary peristalsis and prominent nonpropulsive motor activity during pregnancy. A swallow of water resulted in relief of heartburn simultaneous with the appearance of a normal pressure profile. This might be explained by the offending material having been washed from the esophagus. Secondary peristalsis, induced post partum, was accompanied by similar nonpropulsive motor activity, but no heartburn was present. This suggests that such motor dysfunction may be asymptomatic.

DISCUSSION

Secondary peristalsis, which indirectly indicates that gastroesophageal reflux is taking place, occurred exclusively in a group of women with heartburn during pregnancy, but in only 45 per cent of the group. Neither heartburn nor secondary peristalsis was observed post partum. What evidence is there that secondary peristalsis indicates reflux? By simultaneous manometric and fluoroscopic studies, Creamer demonstrated an association between secondary peristalsis and gastroesophageal reflux (15). The first pressure deflection seen in the secondary peristaltic complexes in our study (Figures 1, 6, 7 and 8) is characteristic of primary peristalsis following a liquid bolus, but is absent during a "dry" swallow. Such a pressure rise is associated with the sudden introduction of material into the esophagus. Since no material was introduced into the esophagus from above during these recordings, the appearance of this initial deflection suggests that material was entering the esophagus from below. It is evident, however, that a certain amount of fluid must distend the esophagus to elicit this response. We introduced 300 ml of water containing a dye

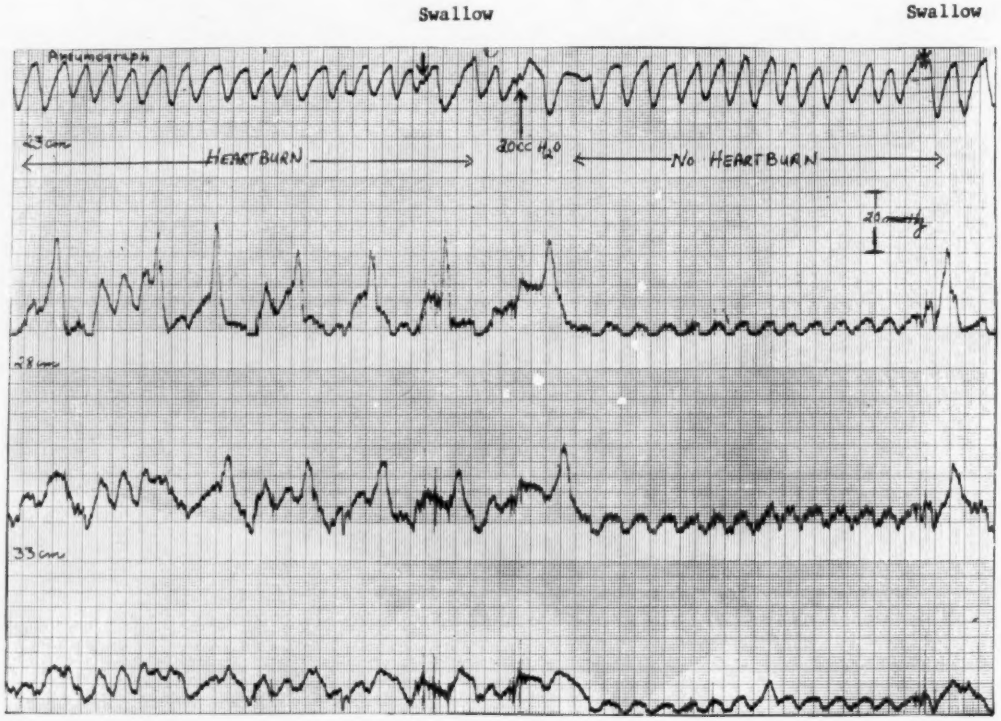


FIG. 8. ELECTROMANOMETRIC RECORDINGS OF SUBJECT 3 (H. C.). The numbers at the left are distances from the incisors. The patient experienced heartburn during the appearance of numerous secondary peristaltic waves. After a swallow of 20 ml of water, the heartburn and the secondary peristalsis simultaneously stopped.

directly into the stomach of 10 control subjects and increased intragastric pressure by inflating a pneumatic abdominal belt. Secondary peristaltic waves appeared in four subjects in whom, at the same time, more than 40 ml of dyed material was aspirated from the distal esophagus. In the other six subjects who did not develop secondary peristaltic waves, less than 10 ml was aspirated from the esophagus, suggesting that the initiation of such a motor response depends upon the regurgitation of relatively large volumes of fluid. We take these results to confirm the relation between secondary peristalsis and gastroesophageal reflux.

The type of material refluxed may bear upon the incidence of heartburn. Reflux occurs in asymptomatic subjects and may be demonstrated during X-ray study of the esophagus and stomach. It is pertinent to note that no symptoms occurred after abdominal pressure was increased by inflation of a pneumatic abdominal belt around the waist in some normal subjects, despite the appearance of secondary peristalsis when 300 ml of water was introduced into the stomach. In contrast, Tuttle, Bettarello and Grossman determined esophageal pressure and intraluminal pH simultaneously; after 300 ml of 0.1 N hydrochloric acid had been introduced into the stomach, heartburn occurred when the pH in the distal esophagus fell below pH 4 (16). In our Patient 1, the reflux of acid appeared, at least from our indirect evidence, to be more important than the mere presence of reflux. Severe heartburn occurred simultaneously with the appearance of secondary peristaltic waves. Although symptoms were relieved with a liquid antacid, the pressure profile remained unchanged. It is true that basal gastric acid is diminished in late pregnancy, and that peptic ulcers are rare during pregnancy (17, 18). Nevertheless, whatever acid and peptic material is available may repeatedly gain access to the esophageal mucosa and over a long period of time may produce esophagitis. In animals, acid gastric juice may devastate esophageal mucosa (19).

A number of factors may contribute to reflux. Increased intragastric pressure is the most obvious: the enlarging uterus allows less room for stomach contents. Many pregnant women experience symptoms of reflux when bending or stooping, actions which result in a sharp rise in intragastric pressure (20). Such a rise in pressure alone might be responsible for reflux, but while the fundal esophageal pressure gradient increased in most pregnant subjects as the uterus enlarged, the women in whom reflux occurred had no higher intragastric pressure than those without reflux, nor did Creamer find that the fundal esophageal pressure gradient rose during spontaneous reflux in nonpregnant subjects (15). Apparently, elevated intragastric pressure must be associated with diminished resistance of the normal barriers if significant reflux is to occur.

Three mechanisms probably help to prevent gastroesophageal reflux: a direct compression or "pinch-cock" action on the esophagogastric junction exerted by the right crus of the diaphragm, an intrinsic sphincter mechanism in the distal esophagus, and a passive mucosal valve formed by the acute angle of entry of the esophagus into the stomach (21, 22). These three barriers probably reinforce each other when properly aligned. We have studied only the sphincteric mechanism.

The variation in the mean resting end-expiratory pressure of the inferior esophageal sphincter from one individual to another is considered by some observers to represent a technical failure of recording methods (23, 24). Serial studies in our control subjects, however, demonstrate that some normal people simply have a well developed intrinsic sphincter mechanism while others do not, but that the tone of the sphincter is relatively constant for the individual. In contrast, in 55 per cent of the pregnant women with heartburn and in 20 per cent of those without heartburn, the resting sphincter pressure decreased with advancing pregnancy and returned to normal levels after delivery. The fall in sphincter pressure may result from gastric atony during pregnancy (25) extending to involve the distal esophagus, which would lower the pressure barrier to reflux. More likely, however, this decrease in resistance to gastroesophageal reflux may be related to loss of the intra-abdominal segment of the sphincter; an intra-abdominal segment was demonstrated in 80 per cent of the controls, but in only 15 per cent of the pregnant women with heartburn and in 37 per cent of those without heartburn. Fyke, Code and Schlegel originally emphasized that an intra-abdominal segment of the sphincter provides an effective barrier to reflux since its intrinsic resistance is supported by the intraperitoneal pres-

sure (2). Further, it has been graphically demonstrated that when intraperitoneal pressure rises, the pressure in the intra-abdominal segment of the sphincter also rises to preserve the normal pressure gradient (26). The intrathoracic segment does not respond with such an increase in pressure, so that if the sphincter is confined to the thorax, as it is in many pregnant women, the normal pressure barrier may be diminished.

In addition, the acute angle of entry of the esophagus into the stomach, so essential in maintaining the flap valve mechanism, is altered by the displacement of the stomach to a more horizontal position by the enlarging uterus (25), and this barrier to reflux may be lost.

A consideration of factors causing gastroesophageal reflux in pregnancy must include hiatus hernia. Three manometric findings are usually interpreted as indicative of hiatus hernia: continuation of intra-abdominal pressures into the thorax, demonstration of the inferior esophageal sphincter more than 3 cm above the effective diaphragmatic hiatus and recognition of a "biphasic" point of respiratory reversal (14, 27, 28). We observed only the latter phenomenon in several women. Since careful roentgenologic examination failed to identify a hiatus hernia in 12 subjects with a biphasic point of respiratory reversal that we have studied, we cannot say with any surety which of the pregnant women had hiatus hernia. Of 195 women in the last trimester of pregnancy, hiatus hernia was radiographically demonstrated in 18.1 per cent of the multiparae and in 5.1 per cent of the primiparae, a combined incidence of 12.8 per cent (29). No relation to heartburn was evident, but those women who showed reflux often had heartburn (29). Although hiatus hernia occurs more often in multiparae, we found no difference in the incidence of secondary peristalsis between primiparae and multiparae. This suggests that while hiatus hernia may have been a factor in some of our patients, it is not an essential factor.

The passage of a tube into the stomach might be responsible for reflux. We have not found a tube to induce reflux by cineradiographic studies of cardioesophageal competence during short-term gastric intubation (30).

Although reflux of an acid may initiate heartburn, the immediate pathogenesis of the symptom is controversial. Muscular dysfunction of the esophagus induced either by distention or by acid reflux may be the final pathway resulting in symptoms of heartburn. Nonpropulsive esophageal pressures developed in patients with heartburn induced by continuous intra-esophageal infusion of 0.1 N hydrochloric acid or equally when heartburn resulted from infusions of other fluids (31). These motor phenomena, however, particularly in the lower two-thirds of the esophagus, are evident in all people, and increase with age (21). Six of 14 asymptomatic young persons whom we have studied demonstrated inconsistently such activity (32). The pregnant women in this study had a marked increase in the frequency and consistency of nonpropulsive responses, but no difference was noted between those women with and without heartburn. The findings in Subjects 2 and 3 are pertinent. Subject 2 demonstrated secondary peristalsis, but nonpropulsive activity did not appear, suggesting that heartburn may occur in its absence. Subject 3 also demonstrated secondary peristalsis, but in addition developed numerous simultaneous nonperistaltic responses representing irritability of the esophagus. When this subject was examined post partum a similar pressure profile was induced but without heartburn, indicating that this motor abnormality may be asymptomatic. Nonpropulsive activity does not appear to be the final common pathway in the pathogenesis of heartburn in pregnancy.

Why pregnant women appear to have an increased frequency of nonpropulsive activity is not clear. Increased intragastric pressure during pregnancy could force air or liquid into the esophagus, which might rapidly transmit pressure along the esophageal lumen so that a pressure response in one area of the organ might be immediately apparent in an adjacent area (33). This is supported by the observation that a normal peristaltic pressure sequence invariably occurred after a swallow of 10 ml of water which could drive air or liquid from the esophagus back into the stomach. In addition, Creamer observed nonpropulsive activity when air was regurgitated into the esophagus (15).

During pregnancy three women with heartburn showed a pressure profile consistent with diffuse esophageal spasm which remained similarly disturbed in the asymptomatic postpartum period.

This does not support a causal relationship. On the other hand, since this pressure profile was observed only in pregnant women with heartburn, it is possible that the abnormal pattern is an indication of motor derangement that with stimulation becomes more profound and produces symptoms.

SUMMARY

Serial esophageal motility studies on pregnant women with and without heartburn and on a group of asymptomatic normal young women of similar age indicate that heartburn of late pregnancy is not determined exclusively by motor changes in the esophagus. Nonpropulsive motor activity was seen consistently and with much greater frequency in the pregnant than in the control subjects, but no difference was noted between pregnant women with heartburn and those without heartburn. It seems likely that such activity is related to pregnancy but not to heartburn.

Diffuse esophageal spasm was found in three pregnant women with heartburn. After delivery this abnormal motor pattern persisted, even though symptoms no longer occurred. It is evident that diffuse esophageal spasm is not in itself responsible for heartburn, but since it occurred in pregnant women with heartburn, it may be an associated abnormality.

Acid reflux into the esophagus appears to be a factor in heartburn of late pregnancy. Secondary peristaltic activity suggests that gastric reflux is taking place into the esophagus and the finding of these complexes exclusively in 45 per cent of pregnant women with heartburn provides indirect evidence to support this concept. Gastroesophageal reflux is favored during pregnancy by a combination of factors. Intraperitoneal pressure rises with advancing pregnancy. The inferior esophageal sphincter, which represents a barrier to reflux, develops progressively weaker resting pressures with advancing pregnancy in more than one-half of the pregnant women with heartburn. The intra-abdominal segment of the sphincter could not be identified in the great majority of these women. Maintenance of this segment appears crucial to sustaining the pressure barrier against reflux especially when intraperitoneal pressure is elevated.

ACKNOWLEDGMENTS

We are grateful to Dr. Massimo Calabresi and his technician, Miss Analee Gelman, for their help in the preliminary stages of this study and to Drs. Clarence D. Davis, Walter Herrmann and Luigi Mastroianni for allowing us to study their patients, and to Mr. Frank Marks and Miss Barbara Van Duzer for their patience and fortitude in the seemingly endless withdrawal of catheters.

REFERENCES

1. Nagler, R., and Spiro, H. M. A prospective clinical study of heartburn in pregnancy. In preparation.
2. Fyke, F. E., Jr., Code, C. F., and Schlegel, J. F. The gastroesophageal sphincter in healthy human beings. Gastroenterologia (Basel) 1956, **86**, 135.
3. Muller Botha, G. S., Astley, R., and Carré, I. J. A combined cineradiographic and manometric study of the gastro-oesophageal junction. Lancet 1957, **1**, 659.
4. Sanchez, G. C., Kramer, P., and Ingelfinger, F. J. Motor mechanisms of the esophagus, particularly of its distal portion. Gastroenterology 1953, **25**, 321.
5. Fleshler, B., Hendrix, T. R., Kramer, P., and Ingelfinger, F. J. Resistance and reflux function of the lower esophageal sphincter. J. appl. Physiol. 1958, **12**, 339.
6. Butin, J. W., Olsen, A. M., Moersch, H. J., and Code, C. F. A study of esophageal pressures in normal persons and patients with cardiospasm. Gastroenterology 1953, **23**, 278.
7. Vantrappen, G., Liemer, M. D., Ikeya, J., Texter, E. C., Jr., and Barborka, C. J. Simultaneous fluorocinematography and intraluminal pressure measurements in the study of esophageal motility. Gastroenterology 1958, **35**, 592.
8. Hightower, N. C., Jr. Swallowing and esophageal motility. Amer. J. dig. Dis. 1958, **3** (n. s.) 562.
9. Hightower, N. C., Jr. Esophageal motility in health and disease. Dis. Chest 1955, **28**, 150.
10. Texter, E. C., Jr., Smith, H. W., Moeller, H. C., and Barborka, C. J. Intraluminal pressures recorded from the upper gastrointestinal tract. I. Correlation with motor activity in normal subjects and patients with esophageal disorders. Gastroenterology 1957, **32**, 1013.
11. Fleshler, B., Hendrix, T. R., Kramer, P., and Ingelfinger, F. J. The characteristics and similarity of primary and secondary peristalsis in the esophagus. J. clin. Invest. 1959, **38**, 110.
12. Siegel, C. I., and Hendrix, T. R. Evidence for central mediation of secondary peristalsis (abstract). Clin. Res. 1960, **8**, 205.
13. Creamer, B., and Schlegel, J. F. Motor responses of the esophagus to distention. J. appl. Physiol. 1957, **10**, 498.

14. Creamer, B., Donaghue, F. E., and Code, C. F. Pattern of esophageal motility in diffuse spasm. Gastroenterology 1958, **34**, 782.
15. Creamer, B. Oesophageal reflux. Lancet 1955, **1**, 279.
16. Tuttle, S. G., Bettarello, A., and Grossman, M. I. Heartburn and gastroesophageal regurgitation (abstract). Clin. Res. 1960, **8**, 100.
17. Spiro, H. M., Schwartz, R. D., and Pilot, M. L. Peptic ulcer in pregnancy. A serial study of gastric secretion during pregnancy. Amer. J. dig. Dis. 1959, **4**, 289.
18. Strauss, M. B., and Castle, W. B. Studies of anemia during pregnancy. I. Gastric secretion in pregnancy and the puerperium. Amer. J. med. Sci. 1932, **184**, 655.
19. Ferguson, D. J., Sanchez-Palomera, E., Sako, Y., Clatworthy, H. W., Jr., Toon, R. W., and Wangensteen, O. W. Studies on experimental esophagitis. Surgery 1950, **28**, 1022.
20. Marchand, P. The gastro-oesophageal sphincter and mechanism of regurgitation. Brit J. Surg. 1955, **42**, 504.
21. Ingelfinger, F. J. Esophageal motility. Physiol. Rev. 1958, **38**, 533.
22. Vantrappen, G., Texter, E. C., Jr., Barborka, C. J., and Vandenbroucke, J. The closing mechanism at the gastroesophageal junction. Amer. J. Med. 1960, **28**, 564.
23. Code, C. F., and Schlegel, J. F. The pressure profile of the gastroesophageal sphincter in man: An improved method of detection. Proc. Mayo Clin. 1958, **33**, 406.
24. Pert, J. H., Davidson, M., Almy, T. P., and Sleisenger, M. H. Esophageal catheterization studies. I. The mechanism of swallowing in normal subjects with particular reference to the vestibule (esophagogastric sphincter). J. clin. Invest. 1959, **38**, 397.
25. Williams, N. H. Variable significance of heartburn. Amer. J. Obstet. Gynec. 1941, **42**, 814.
26. Nagler, R., and Spiro, H. M. Segmental response of the inferior esophageal sphincter to elevated intragastric pressure. Gastroenterology 1961. In press.
27. Atkinson, M., Edwards, D. A. W., Honour, A. J., and Rowlands, E. N. The oesophagogastric sphincter in hiatus hernia. Lancet 1957, **2**, 1138.
28. Texter, E. C., Jr., Lazar, H. P., Puletti, E. J., and Vantrappen, G. The characteristic pattern of esophageal dysfunction due to hiatal hernia demonstrated by fluorocinematography and simultaneous pressure recording (abstract). J. clin. Invest. 1959, **38**, 1048.
29. Rigler, L. G., and Eneboe, J. B. The incidence of hiatus hernia in pregnancy and its significance. J. thorac. Surg. 1934, **4**, 262.
30. Nagler, R., Wolfsohn, A. W., Lowman, R. M., and Spiro, H. M. The effect of gastric intubation on the normal mechanisms preventing gastro-esophageal reflux. New Engl. J. Med. 1960, **262**, 1325.
31. Lazar, H. P., Puletti, E. J., Douglas, W. W., Danovitch, S., and Texter, E. C., Jr. Nonperistaltic esophageal motility accompanying experimentally produced heartburn (abstract). J. Lab. clin. Med. 1959, **54**, 917.
32. Nagler, R., and Spiro, H. M. Serial esophageal motility studies in normal young subjects. In preparation.
33. Ingelfinger, F. J. Personal communication.

THE RELATIONSHIP OF OXYGEN COST OF BREATHING TO RESPIRATORY MECHANICAL WORK AND RESPIRATORY FORCE

By MAURICE McGREGOR * AND MARGARET R. BECKLAKE

(From the Joint Cardio-respiratory Service of the Royal Victoria Hospital and the Montreal Children's Hospital, Department of Medicine, McGill University, Montreal, Canada)

(Submitted for publication January 25, 1960; accepted February 16, 1961)

The oxygen cost of increased respiratory activity can be measured by subtracting the oxygen consumption at rest from that observed during the increased respiratory activity. The efficiency of the system can be assessed when the increased respiratory mechanical work is related to the oxygen cost.

Previous estimates of efficiency in man have varied very widely, possibly because very different forms of respiratory work have been employed. Some workers have increased the pressure component of respiratory work by additions to the external air flow resistance, a procedure which will be referred to hereafter as "resistance breathing." In other studies, the volume component of respiratory work has been augmented by increasing the minute volume, without any addition of airway resistance: this will be referred to as "hyperventilation." No investigator employing one experimental technique has studied the oxygen cost of both of these forms of activity.

The first objective of this study was to ascertain whether the relationship of oxygen cost to mechanical respiratory work is the same for hyperventilation as for resistance breathing in normal subjects. The second was to test whether the same relationships hold in the presence of diseases which alter the mechanical properties of the lung.

There is evidence, however, that mechanical work is not the most revealing parameter with which to compare energy consumption. For example, the support of a heavy object at a fixed distance from the ground involves energy consumption but results in no measurable mechanical work, although it is likely that the increased energy consumption would relate to the weight of the object and the length of time it was supported. It was considered of interest to establish the relationship of oxygen consumption of the respiratory muscles to the force exerted by them on the lung.

METHOD

Five healthy volunteer sedentary workers, 5 subjects with obstructive respiratory disease, and 4 cardiac patients were selected for study. Selection of patients was determined by the presence of abnormalities of the mechanical properties of the lungs (Table I). All the patients with obstructive lung disease had moderately severe impairment of exercise capacity except Subject 6 in whom impairment was mild. The two subjects with mitral stenosis had moderate functional disability meriting surgical intervention, while the patients with mitral regurgitation and atrial septal defect suffered from no more than minimal limitation of exercise tolerance. The physical characteristics and results of routine lung function tests are listed in Table I.

Pressure was measured in the esophagus with an esophageal balloon and differential transformer manometer [1] with subjects seated and leaning slightly forward (4). Although usually adjusted to record the difference between esophageal and atmospheric pressure, transpulmonary pressure (differential pressure between esophagus and mouth) also could be observed (see Figure 1). It was assumed that the esophageal balloon recorded the instantaneous average pressure over the outer surface of both lungs.

Respiratory volume was recorded with a sensitive spirometer [2] coupled to a potentiometer, the output of which, together with that of the esophageal pressure gauge, was recorded on a laboratory polygraph. The circuit (Figure 1) consisted of a mouthpiece and low-resistance box valve (5) connected to the oxygen-filled spirometer, so that during control observations all of the expired gas passed through the soda-lime absorber. During hyperventilation an adjustment to the three-way stopcock, T_1, deflected expired gas to the inspiratory line so that expired gas was now partially rebreathed. By selection of a dead space of appropriate size, overbreathing could be achieved without discomfort or faintness resulting from hypocapnea. During resistance breathing the continuity of the circuit was restored, but stopcocks T_1 and T_2

* Research Associate of the National Research Council of Canada.

[1] No. 267B, Sanborn Company, Dover, Mass.

[2] Stead-Wells, Warren E. Collins, Inc., Boston, Mass.

TABLE I

Physical characteristics and respiratory function values *

Subj.	Sex	Diagnosis	Age	Ht	Wt	MMF	Mix.	FRC	VC	Comp.	Resistance In	Resistance Out
			yrs	*cm*	*kg*	*L/sec*	*%*	*L*	*L*	*L/cm H_2O*	*cm H_2O/L/sec*	
1	M	Normal	39	175	73	3.84	74	4.58	4.22	0.17	1.77	2.29
2	M	Normal	44	173	75	2.80	52	2.91	4.45	0.25	4.20	3.50
3	M	Normal	34	178	73	3.48	77	3.35	5.30	0.28	2.97	4.20
4	M	Normal	30	179	80	4.58	68	3.12	4.64	0.18	2.25	2.72
5	F	Normal	28	160	53	4.70	50	2.45	2.92	0.13	0.91	0.96
6	M	Emphysema	66	166	63	0.21	35	3.74	1.58	0.25	6.30	8.90
7	M	Emphysema	49	159	47	0.15	35	5.92	1.47	0.20	11.80	19.80
8	M	Emphysema	59	173	67	0.30	30	2.56	2.02	0.18	10.00	21.80
9	M	Asthma	50	168	69	0.30	32	6.32	1.92	0.10	15.10	21.30
10	F	Asthma	44	157	66	0.48	57	1.76	1.70	0.08	10.80	17.30
11	F	MS	51	163	42	0.40	56	3.07	1.53	0.11	5.50	9.92
12	F	MS	31	161	67			2.60		0.05	9.18	20.10
13	M	MI	19	178	70	5.70	49	2.83	3.94	0.11	3.80	6.60
14	F	ASD	22	155	35	2.07	77	2.42	2.20	0.06	6.10	12.50

* MS = mitral stenosis;
ASD = inter-atrial septal defect;
MI = mitral incompetence;
MMF = maximal mid-expiratory flow (1);
Mix. = mixing index (2);
FRC = functional residual capacity (2); VC = vital capacity;
Comp. = pulmonary compliance (3).

were adjusted so that inspired and expired gas had to pass through the resistances.

Pressure flow characteristics of the apparatus are illustrated in Figure 2. All tubing was of 32 mm internal bore; thus, resistance to flow was small during control observations and hyperventilation. During resistance breathing the added resistances consisted of lengths (32 cm and 79 cm) of rubber tubing of 5.0 mm internal bore.

Method of procedure was as follows. After a fast of at least 4 hours, the subjects rested in a chair for 30 minutes, breathed pure oxygen for 5 minutes, and then underwent initial control observations for 8 to 12 minutes.

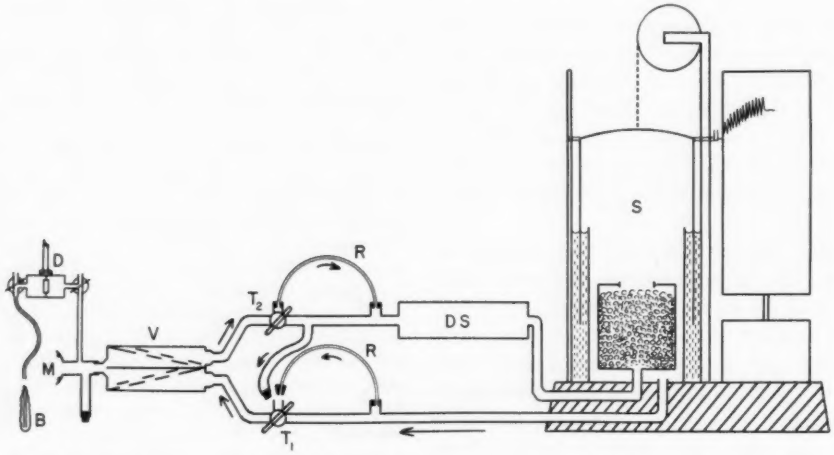


FIG. 1. THE APPARATUS. D = differential manometer; M = mouthpiece and sputum trap; V = low resistance box valve (5); DS = adjustable deadspace; S = Stead-Wells spirometer, with soda-lime absorber; R = variable resistances; B = esophageal balloon; T_1, T_2 = wide-bore three-way stopcocks.

Thereafter, increased respiratory activity was initiated in one of the two ways mentioned. For hyperventilation the subject voluntarily increased his ventilation for 1 to 3 minutes, the apparatus being adjusted for rebreathing. Continuity of the circuit was then restored and control observations again made for a further 8 to 12 minutes. Resistance breathing was likewise preceded and followed by periods of control observation. During the 1 to 3 minutes during which the normal subjects inspired and expired through added resistances, they were instructed to use sufficient effort to maintain the ventilatory volume at normal levels. Unlike the normal subjects it was found that none of the patients could tolerate resistance breathing.

Oxygen consumption during control periods before and after the period of increased ventilatory activity was derived from the slope of the spirometer tracing. The lines describing these slopes, although parallel, do not meet when projected through the period of increased respiratory work but are separated by a distance equivalent to the additional oxygen consumed by the increased work (6, 7). When the oxygen consumption of the second control period differs from the first, the lines describing each are not parallel and the distance between them can no longer be used to determine the increase of oxygen uptake during the period of increased respiratory work. An arbitrary decision was made to exclude all studies in which the control oxygen uptake values before and after the period of increased respiratory activity differed by more than 20 ml per minute. Oxygen uptake values were expressed as STPD, lung volume measurements as APTS, and the breath volume used for computing the respiratory mechanical work was measured at room temperature and pressure.

Mechanical respiratory work per breath was estimated from the area of the loop which resulted from plotting instantaneous esophageal pressure and volume with the addition of that portion of a triangle describing work against lung elastic forces which fell outside this loop. The base of the triangle describing the elastic forces was plotted at the end-expiratory pressure level, not, as is usual, at atmospheric pressure. Because data are based on *differences* between control periods and periods of increased respiratory work, this method of plotting the pressure volume diagram seemed permissible and had the advantage of avoiding the considerable error which may result when *absolute* levels of pleural pressure, as distinct from respiratory fluctuation, are derived from esophageal pressure measurements (3). The product of the average work for 6 to 12 breaths and the frequency gave the respiratory work expressed as kilogram-meters per minute.

The work thus measured, therefore, represents the work performed by the chest cage and diaphragm on the lung and its contents. It includes the work of the abdominal and other accessory muscles during increased work. It does not include the work of the respiratory muscles on the chest cage itself and on the abdominal viscera.

The efficiency of this system was estimated by expressing the increase in mechanical respiratory work, as defined above, as a percentage of the energy equivalent (kilogram-meters per minute) of its increased oxygen consumption.[3]

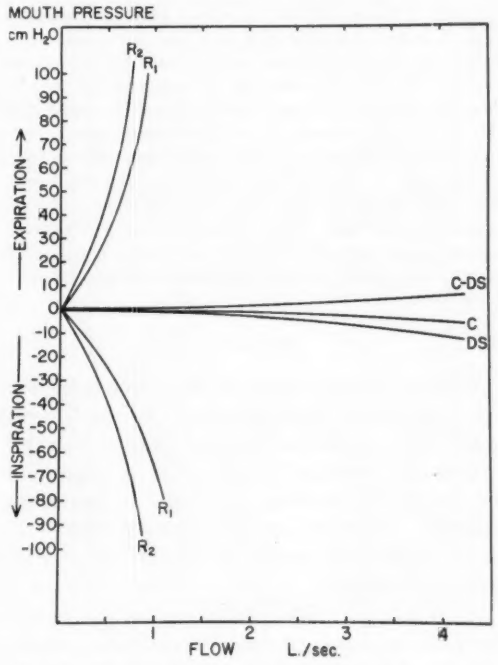


FIG. 2. PRESSURE FLOW RELATIONSHIPS FOR THE INSTRUMENT UNDER CONDITIONS OF USE. C = control observations; DS = hyperventilation through deadspace; R_1, R_2 = resistance breathing. During expiration, C and DS coincide.

Respiratory force was defined as the force exerted, at any instant, by the chest and abdominal muscles on the lung surface, the area of which includes the diaphragmatic and thoracic surfaces of both lungs, but not the mediastinal surface. It was impossible to measure this area but, as a first approximation, it was assumed to equal the outer surface of a solid hemisphere equal in volume to the functional residual capacity. Force per breath was obtained by the product of the mean esophageal pressure per breath (as defined later) and this area. The resultant values depend too greatly upon approximations to be accepted as defining with precision, in kilograms, the actual force exerted. However, inferences based on changes in these values rather than on the values themselves are valid.

Esophageal pressure per breath was measured from the pressure curve. For this purpose it was assumed that the end-expiratory esophageal pressure was the result of the balanced elastic recoil of the chest and lung; change of pressure from this value resulted from the application

[3] A respiratory quotient of 0.8 was assumed.

of force by the respiratory muscles. Change of esophageal pressure from this reference level (irrespective of sign) was thus integrated against time for the whole breath. It will be referred to subsequently as the "mean esophageal pressure" and it should be noted that this term does not refer to an arithmetical mean. Control pressure values and work loops were derived from the average of 6 to 12 breaths in the two periods of resting breathing before and after the period of increased respiratory activity. Values during increased respiratory activity were averaged from 6 to 12 breaths during each of the 1 to 3 minutes of hyperventilation or resistance breathing.

RESULTS

During resting ventilation the average respiratory mechanical work performed by the patients with lung disease was 2.7 times greater than that performed by normal subjects. The average value for the cardiac subjects was similar to that of the normals. Values for respiratory force showed the same trends with greater separation between the groups (Table II).

During hyperventilation the normal and cardiac subjects achieved greater increments of ventilation at a lower oxygen cost per liter ventilated than did the patients with lung disease. Average values for normal and cardiac subjects were 2.23 and 2.26 ml oxygen per liter ventilation, respectively. The equivalent value for patients with lung disease was 21.43 ml per L (Table III). These, with one exception, failed to double their resting ventilatory level in spite of considerable effort. The oxygen cost per kilogram-meter respiratory mechanical work was, on the average, twice as great in the patients with chest disease. Average efficiency as defined for normal, cardiac and respiratory subjects was 3.2, 3.4 and 1.6 per cent, respectively. When related to respiratory force, however, with the exception of Patient 6, the oxygen cost per kilogram was similar in the three groups.

Resistance breathing, which could only be performed by the normal subjects, gave results which were strikingly different from those obtained during hyperventilation (Table IV). The oxygen cost per kilogram-meter respiratory mechanical work was higher during resistance breathing (average efficiency 1 per cent). By contrast, the cost per kilogram respiratory force for this form of activity was lower.

When the data relating oxygen cost to respiratory work for normal and cardiac subjects were plotted (Figure 3A) it was apparent that they were indistinguishable, falling in two distinct slopes for resistance breathing and hyperventila-

TABLE II

Average resting (control) observations *

Subj.	Diagnosis	No. of obs.	f		V_T		$\dot{V}$		R. force		R. mech. work	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
							L/min		*kg*		*kg-m/min*	
1	Normal	12	15.9	0.99	0.58	0.030	9.3	0.99	1.50	0.276	0.22	0.034
2	Normal	12	12.5	0.42	0.95	0.246	11.6	1.92	1.76	0.667	0.54	0.253
3	Normal	6	10.9	1.66	1.03	0.182	10.9	1.07	1.41	0.608	0.44	0.123
4	Normal	6	14.6	0.79	0.77	0.047	11.2	1.01	1.55	0.110	0.35	0.014
5	Normal	6	16.5	1.93	0.47	0.023	7.7	0.73	1.26	0.141	0.75	0.664
	Mean		14.08		0.76		10.14		1.50		0.46	
6	Emph.	2	20.0		0.40		8.0		5.60		0.71	
7	Emph.	2	17.3		0.51		8.8		7.93		1.23	
8	Emph. fib.	2	19.0		0.60		11.4		5.86		1.65	
9	Asthma	4	16.0	0.58	0.68	0.095	12.0	1.09	10.80	0.024	1.66	
10	Asthma	2	13.0		0.48		6.2		4.82		1.16	
	Mean		17.06		0.53		9.28		7.00		1.28	
11	MS	2	14.0		0.53		7.4		3.27		0.51	
12	MS	2	12.0		0.60		7.2		2.94		0.49	
13	MI	2	24.0		0.48		11.5		1.74		0.41	
14	ASD	4	19.0	1.15	0.85	0.046	8.7	0.340	1.71	0.208	1.00	
	Mean		17.25		0.62		8.70		2.41		0.60	

* f = Respiratory frequency; V_T = tidal volume.

TABLE III

Changes in ventilation, oxygen uptake, respiratory mechanical work and respiratory force resulting from unobstructed hyperventilation in all subjects

Subj.	Diagnosis	f	V_T	$\Delta \dot{V}$	Δ R. mech. work	Δ R. force	$\Delta \dot{V}_{O_2}$	Oxygen cost		
									R. mech. work	R. force
				L/min	*kg-m/min*	*kg/min*	*L/min*	*ml O_2/L vent.*	*ml O_2/kg-m*	*ml O_2/kg*
1	Normal	23	1.11	16.4	1.14	2.10	0.022	1.34	19.30	10.48
		21	1.59	22.3	2.77	3.68	0.035	1.57	12.64	9.51
		27	1.66	36.2	2.28	4.97	0.048	1.33	21.05	9.66
2	Normal	13	3.50	34.9	9.30	12.26	0.150	4.30	16.12	12.23
		18	2.83	36.0	21.34	17.47	0.200	5.55	9.37	11.45
		120	0.70	71.6	55.96	31.00	0.313	4.40	5.59	10.10
3	Normal	14	0.52	21.6	1.61	4.34	0.047	2.18	29.19	10.83
		16	1.35	24.2	2.84	5.72	0.055	2.27	19.36	9.62
4	Normal	27	2.31	50.4	10.70	9.59	0.115	2.28	10.75	11.99
5	Normal	23	1.80	34.2	14.79	12.04	0.128	3.74	8.65	10.63
		43	1.40	51.4	13.09	11.70	0.175	3.40	13.37	14.96
	Mean							2.23	15.04	11.05
6	Emphysema	25	0.91	14.9	2.20	2.83	0.072	4.90	32.73	25.44
7	Emphysema	15	0.82	3.5	4.15	24.07	0.230	65.71	55.42	9.55
8	Emphysema	23	0.85	8.1	12.05	31.55	0.202	24.93	16.76	6.40
9	Asthma	40	0.69	16.6	4.56	13.68	0.158	9.52	34.65	11.55
		42	0.57	10.0	6.63	24.58	0.197	19.70	29.71	8.01
10	Asthma	25	1.00	19.0	5.82	5.27	0.073	3.84	12.54	13.85
	Mean							21.43	30.47	12.47
11	MS	30	1.00	22.6	4.87	6.60	0.045	1.99	9.24	6.82
12	MS	28	1.20	26.4	6.76	5.00	0.069	2.61	10.20	13.80
13	MI	32	1.40	33.3	7.64	6.38	0.085	2.55	11.13	13.32
14	ASD	46	1.12	43.0	3.08	6.33	0.081	1.88	26.30	12.80
	Mean							2.26	14.22	11.69

TABLE IV

Changes in ventilation, oxygen uptake, respiratory mechanical work and respiratory force resulting from resistance breathing in normal subjects

Subj.	f	V_T	$\Delta \dot{V}$	Δ R. mech. work	Δ R. force	$\Delta \dot{V}_{O_2}$	Oxygen cost	
							R. mech. work	R. force
			L/min	*kg-m/min*	*kg/min*	*L/min*	*ml O_2/kg-m*	*ml O_2/kg*
1	15	0.71	2.2	2.08	13.10	0.070	33.65	5.34
	17	0.80	4.4	2.43	29.03	0.131	53.90	4.51
	16	0.74	3.7	5.05	25.29	0.153	30.30	6.04
2	6	2.15	1.2	3.26	16.41	0.120	36.81	7.31
	8	1.84	7.5	7.33	28.33	0.216	29.47	7.62
	18	0.55	−4.1	7.89	31.80	0.304	38.53	9.56
3	10	0.80	−2.7	0.18	5.13	0.033	183.30	6.43
	6	1.28	−4.2	1.10	10.97	0.065	59.09	5.93
4	20	0.70	3.7	6.57	26.95	0.139	21.16	5.16
	13	0.91	−0.2	5.21	28.68	0.150	28.79	5.23
5	12	0.66	−0.6	2.86	9.66	0.088	30.77	9.11
Mean							49.62	6.57

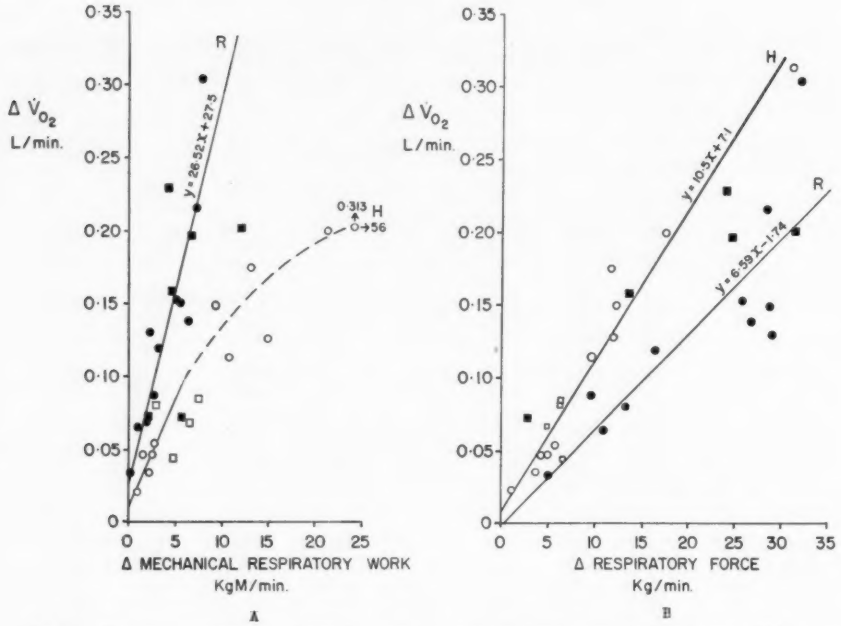


FIG. 3. THE RELATIONSHIP OF OXYGEN COST TO RESPIRATORY MECHANICAL WORK (A) AND TO AN INDEX OF RESPIRATORY FORCE (B). The regression equations are based on the data for normal subjects. ○ = normal subjects during hyperventilation (H); □ = patients with heart disease during hyperventilation; ● = normal subjects during resistance breathing (R); ■ = patients with obstructive lung disease during hyperventilation.

tion, respectively. The patients with obstructive lung disease, however, who in spite of attempted hyperventilation could achieve little increase in ventilatory volume, gave values comparable to those of the normal subjects performing resistance breathing. When oxygen cost was plotted against force (Figure 3B) the normal and cardiac subjects were again comparable, although resistance breathing was achieved at a lower cost than hyperventilation. The data for the patients with lung disease were widely scattered.

DISCUSSION

A possible objection to the validity of these measurements is that major changes in lung volume during the period of increased respiratory activity would tend to alter the area of lung over which pressure was being exerted, and hence produce error in the computation of "force." As judged by shifts in the end-expiratory level of the spirometer tracing, however, these changes were not great. During hyperventilation, normal and cardiac subjects behaved similarly with an average reduction of functional residual capacity of 260 ml (range, 100 to 500 ml). As might be expected, the subjects with obstructive pulmonary disease tended to trap air during hyperventilation with an average increase of functional residual capacity of 200 ml (range, − 100 to + 600 ml). During resistance breathing the end-expiratory level tended to change from breath to breath, the average change in functional residual capacity in normal subjects being an increase of 111 ml (range, − 400 to + 600 ml).

Previous estimations of the efficiency of respiration in normal subjects have shown considerable variation, from as low as 1 per cent (6) to as high as 25 per cent (8). Such discrepancies may be due to differences in the methods of measurement of oxygen cost and of respiratory mechanical work and to differences in the nature of increased respiratory activity.

In most previous studies the oxygen uptake has been measured during prolonged periods (7 to 40 minutes) of increased respiratory activity aimed

at achieving a steady state (8–10). Others, as in the present study, have used short (1 to 3 minutes) bursts of increased respiratory activity (6, 7). The latter method makes possible the study of much higher levels of respiratory work, and there is no reason to believe the results are less accurate. Thus, the oxygen cost observed in this study, when compared with ventilatory volume, is comparable to the values obtained by steady state methods for equivalent degrees of hyperventilation (9–11). Therefore, divergent values for efficiency are unlikely to be due largely to differences in measuring the oxygen uptake—they are more likely due to differences in the method of measurement of respiratory mechanical work.

The efficiency of the combined chest and lung for external resistance work, assessed in three studies (6, 7, 10), gave results of 1 to 10 per cent. In two studies (8, 9), respiratory mechanical work estimates during unobstructed hyperventilation were derived from the area of loops obtained by plotting esophageal pressure against respiratory volume, as in the present study. The values for efficiency for hyperventilating normal subjects obtained in the present study (average, 3.19 per cent) agreed with those in one of these reports (9) but are well below those (19 to 25 per cent) reported in the other (8). A possible reason for this discrepancy is the use of the supine posture by the latter workers. Mead and Gaensler (3) have reported that the esophageal pressure fluctuation may exceed the simultaneous pleural pressure by as much as 60 per cent in the supine position, an artifact which might lead to very considerable overestimation of the mechanical respiratory work.

Irrespective of the absolute values for efficiency obtained in this study, however, it is clear that when the same method is employed for estimating efficiency during both hyperventilation and resistance breathing, the efficiency during the former activity is greater. It should be recalled, however, that in the present study the efficiency of the chest as a whole has been considered. Mechanical work measured did not include that of moving the chest cage or the abdominal contents. For resistance breathing, during which resting ventilatory levels were maintained, there would be no significant increase in the energy expended in moving the chest and abdominal structures so that the calculated efficiency would be unaffected. During hyperventilation, however, unmeasured work was performed on these structures, resulting in an underestimate of the efficiency of the respiratory muscles. If this additional mechanical work could be measured and included in the estimation of efficiency, the discrepancy between resistance breathing and hyperventilation would be even further increased. Thus, in Figure 3A the resistance plots would be unchanged, but those representing hyperventilation would be displaced further to the right.

Consideration of the data for respiratory force, however, is very different. This term likewise described the force applied by the intact chest, diaphragm and abdominal muscles to the lung's surface. Force supplied by the respiratory muscles to the ribs and abdominal structures was not measured. As before, this omission would not affect the relationship of force to oxygen uptake during resistance breathing, but would cause an underestimate of the force exerted by the respiratory muscles during hyperventilation.

If the force exerted by the respiratory muscles on the chest and abdominal structures could be measured and included, the line in Figure 3B describing observations made during hyperventilation would be displaced to the right; that is, nearer to the line describing resistance breathing. In fact, it seemed possible that the discrepancy between the regression equations describing resistance breathing and hyperventilation might be an index of the oxygen cost of moving the chest wall itself.

To check the validity of this hypothesis in Subject 1, measurements were made of the compliance and resistance of the chest wall and lung both separately and together.[4] From the instantaneous volume and flow values, the pressure necessary to deflect the chest wall was computed from instant to instant and added with the appropriate sign to the esophageal pressures recorded in the previous studies. The resulting data for this subject are plotted in Figure 4. In Figure 4A, it can be seen again that the oxygen cost of hyperventilation per

[4] Chest wall compliance = 0.190 ml per cm H_2O; chest wall resistance = 0.6 cm per H_2O per L per second. Measurements were kindly made by Dr. Jere Mead at the Harvard School of Public Health, by a technique reported elsewhere (12).

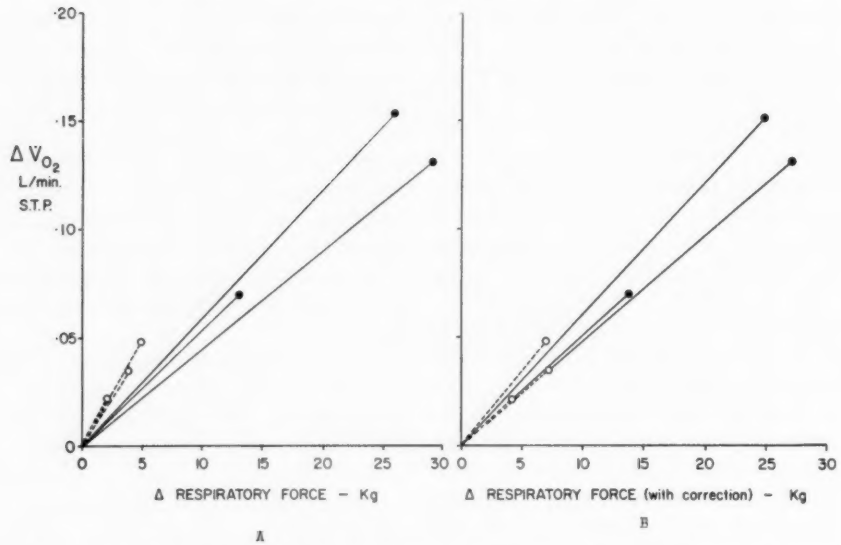


FIG. 4. A) THE RELATIONSHIP OF OXYGEN COST TO RESPIRATORY FORCE EXERTED ON THE LUNG IN SUBJECT 1. B) THE RELATIONSHIP OF OXYGEN COST TO RESPIRATORY FORCE EXERTED ON THE LUNG WITH THE ADDITION OF THE ESTIMATED FORCE EXERTED ON THE CHEST WALL IN SUBJECT 1. ○ = hyperventilation; ● = resistance breathing.

unit force appears greater than that of resistance breathing. With addition of the component of force estimated to be necessary to move the chest wall, the slopes for resistance breathing and hyperventilation cannot be distinguished (Figure 4B).

This suggests that the relationship between oxygen cost and respiratory muscle force may be represented by two slopes which will diverge according to the amount of force which is exerted against the chest and abdominal structures. In other words, the divergence between these slopes (Figure 3B) may represent the additional energy cost of moving the chest and abdominal structures during hyperventilation. No such explanation, however, could account for the differences observed in oxygen cost per unit respiratory mechanical work for the two forms of respiratory activity (Figure 3A).

The observations made on patients with heart and lung disease are too few for any but tentative conclusions. The oxygen cost of hyperventilation in the subjects with heart disease was indistinguishable from that of normal subjects in spite of the fact that the cardiac subjects, although not severely incapacitated, had detectable abnormalities of lung mechanics (Table I). It is likely, however, that heart disease of sufficient severity must result in a significant elevation of respiratory oxygen cost, and Cournand and co-workers did demonstrate this in a comparison of one normal subject with one patient suffering from tight mitral stenosis (13). In the presence of obstructive lung disease, however, the oxygen cost of hyperventilation was very high, due primarily to the high levels of mechanical respiratory work necessary for the small ventilatory increase. It was also due in part to the fact that the oxygen cost per unit mechanical respiratory work was increased above normal. Low respiratory efficiency in emphysematous subjects has been reported previously by Cherniack (10) and is supported by the figures of Fritts, Filler, Fishman and Cournand (9), although they did not consider the values to be "greatly different" from normal. There are two possible reasons for the finding of a reduced mechanical efficiency in these subjects. One is inherent in the method by which mechanical work is computed, i.e., the product of esophageal pressure and volume of gas moved. High levels of obstruction of the airway must result in reduction of the ratio of work measured to energy consumed.

As an extreme example, in maximal respiratory effort against a completely obstructed airway the oxygen consumption will be great, mechanical work measured nil, and efficiency, thus, zero. There is no reason, however, for the force measured to fall off in this way even with complete airway obstruction.

An alternative reason for the low mechanical efficiency measured in the subjects with obstructive lung disease is that their respiratory muscles were acting at a mechanical disadvantage due to hyperinflation. Consideration of the force data provides some support for this (Figure 3B). These subjects, while attempting hyperventilation, failed to increase their minute ventilation greatly in spite of considerable effort (solid squares, Figure 3B), and were thus, in effect, performing "resistance" breathing. The oxygen cost per unit force, however, was apparently higher in most instances than that of normal subjects breathing against increased resistance. These findings indicate an increased oxygen consumption per unit force developed and would be consistent with the working of the respiratory muscles at mechanical disadvantage.

The present findings suggest that as a parameter for comparison with energy consumption, force is a more meaningful expression than mechanical work. A similar conclusion has been reached for heart muscle. Thus, it has been shown that, although heart work augmented by the elevation of arterial pressure consumes more oxygen than does work increased by augmenting venous return, the product of force developed and time appears to relate in a linear fashion to myocardial oxygen consumption over a wide range (14–16) whatever method is used to increase heart work.

Force may also relate more significantly than mechanical work to other respiratory parameters. Marshall, Stone and Christie (17), comparing the onset of severe dyspnea in normal, emphysematous and mitral stenotic subjects, found that, although the level of mechanical respiratory work at which dyspnea became limiting differed greatly in the three groups, exercise was curtailed by dyspnea at a comparable level of "respiratory force" in each group. Furthermore, Mead (12) has recently demonstrated that respiratory depth and frequency vary, not according to the minimal respiratory mechanical work necessary to secure alveolar ventilation, but rather with the minimal respiratory force.

Finally, it is clear that the high oxygen cost of hyperventilation in obstructive lung disease is not only due to increased work of breathing but also to an increased oxygen cost per unit work. This is unlikely to be an artifact in the measurement of respiratory work since the oxygen cost per unit force is also high in these subjects.

SUMMARY

In five normal subjects the oxygen cost of breathing was measured during two forms of increased respiratory activity: unobstructed hyperventilation and breathing in the presence of a high airway resistance. Oxygen cost per unit mechanical respiratory work was greater for resistance breathing than for hyperventilation. Inclusion of the work performed on the chest wall would have increased this anomaly.

By contrast, when related to an index of respiratory force, oxygen cost was higher for hyperventilation. When allowance was made for the force necessary to deflect the chest wall in one subject, the relationship between force and oxygen cost was similar for both forms of respiratory activity. Thus, force appeared to be a more logical parameter to compare with energy consumption than was mechanical work.

In four subjects with disturbances of respiratory mechanics due to heart disease the oxygen cost of hyperventilation was normal whether related to ventilatory volume, respiratory mechanical work or respiratory force.

In five subjects with obstructive lung disease the oxygen cost of hyperventilation was high. This was due both to increased mechanical work per unit volume ventilated and to reduced efficiency per unit mechanical respiratory work. Since oxygen cost per unit force was also high it was likely that the low efficiency was the result of the respiratory muscles' working at a mechanical disadvantage.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the very great theoretical and practical assistance they have received during this study in particular from Drs. Ronald V. Christie and Jere Mead. They gratefully acknowledge the assistance of Dr. R. Kamener, Mrs. J. O'Dono-

van, Dr. R. Donevan, Dr. C. Varvis, Mrs. M. Pablo and Mr. W. Fowler in the performance of these experiments and of Dr. David V. Bates in the preparation of this manuscript. Finally, the kind cooperation of their subjects in an uncomfortable experiment is most gratefully acknowledged.

REFERENCES

1. Leuallen, E. C., and Fowler, W. S. Maximal mid-expiratory flow. Amer. Rev. Tuberc. 1955, **72**, 783.
2. Bates, D. V., and Christie, R. V. Intrapulmonary mixing of helium in health and in emphysema. Clin. Sci. 1950, **9**, 17.
3. Mead, J., and Gaensler, E. A. Espohageal and pleural pressures in man, upright and supine. J. appl. Physiol. 1959, **14**, 81.
4. Mead, J., and Whittenberger, J. L. Physical properties of human lungs measured during spontaneous respiration. J. appl. Physiol. 1953, **5**, 779.
5. Theron, J. C., Zwi, S., and McGregor, M. A low-resistance respiratory valve. Lancet 1958, **1**, 415.
6. Cain, C. C., and Otis, A. B. Some physiological effects resulting from added resistance to respiration. J. Aviat. Med. 1949, **20**, 149.
7. Bartlett, R. G., Jr., Brubach, H. F., and Specht, H. Oxygen cost of breathing. J. appl. Physiol. 1958, **12**, 413.
8. Milic-Emili, G., and Petit, J. M. Mechanical efficiency of breathing. J. appl. Physiol. 1960, **15**, 359.
9. Fritts, H. W., Jr., Filler, J., Fishman, A. P., and Cournand, A. The efficiency of ventilation during voluntary hypernea: Studies in normal subjects and in dyspneic patients with either chronic pulmonary emphysema or obesity. J. clin. Invest. 1959, **38**, 1339.
10. Cherniack, R. M. The oxygen consumption and efficiency of the respiratory muscles in health and emphysema. J. clin. Invest. 1959, **38**, 494.
11. Murray, J. F. Oxygen cost of voluntary hyperventilation. J. appl. Physiol. 1959, **14**, 187.
12. Mead, J. Control of respiratory frequency. J. appl. Physiol. 1960, **15**, 325.
13. Cournand, A., Richards, D. W., Jr., Bader, R. A., Bader, M. E., and Fishman, A. P. The oxygen cost of breathing. Trans. Ass. Amer. Phycns 1954, **67**, 162.
14. Sarnoff, S. J., Braunwald, E., Welch, G. H., Jr., Case, R. B., Stainsby, W. N., and Macruz, R. Hemodynamic determinants of oxygen consumption of the heart with special reference to the tension-time index. Amer. J. Physiol. 1958, **192**, 148.
15. Lendrum, B., Feinberg, H., and Katz, L. N. Isometric left ventricular contraction. Fed. Proc. 1959, **18**, 89.
16. Salisbury, P. F., and Bor, N. Hemodynamic determinants of cardiac energy transformation; their correlation with cardiac oxygen supply and with coronary flow. Fed. Proc. 1959, **18**, 134.
17. Marshall, R., Stone, R. W., and Christie, R. V. The relationship of dyspnoea to respiratory effort in normal subjects, mitral stenosis and emphysema. Clin. Sci. 1954, **13**, 625.

A LIVER ENZYME THAT CONJUGATES SULFOBROMOPHTHALEIN SODIUM WITH GLUTATHIONE*

By BURTON COMBES† AND GENEVA SUE STAKELUM

(From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas)

(Submitted for publication December 2, 1960; accepted February 10, 1961)

In the past few years, considerable evidence has been presented indicating that sulfobromophthalein sodium (BSP) is metabolized in the liver (1–6). Recently, we have demonstrated that the major pathway of BSP metabolism in man and in the rat involves conjugation of BSP with the tripeptide glutathione (5). Similar results have been obtained by Javitt and his associates in the dog (6). Grodsky, Carbone and Fanska concluded "that BSP is excreted at least in part as a mercaptide with cysteine or the peptide glutathione," in man (4). These latter authors are not certain about the presence of glutathione, however, since they feel that glycine and glutamic acid are possible contaminants of the BSP metabolites.

The results of the present investigation disclosed three critical features of hepatic BSP metabolism; first, an enzyme is described, identified in liver, which catalyzes the conjugation of BSP and glutathione; second, glutathione is shown to be the preferred substrate for the enzyme; finally, it is demonstrated that 1 mole of bromide ion is released from BSP for each mole of BSP-glutathione formed.

METHODS

1. Preparation of liver homogenates and subcellular fractions. Adult, male, Sprague-Dawley rats were stunned by a blow on the head, their throats cut, and their livers perfused through the portal vein with 20 ml of ice-cold phosphate buffer, 0.1 M, pH 7.8. The livers were then excised and placed in cold buffer to cool. After weighing, the livers were homogenized in a Dounce homogenizer (7) with a volume of phosphate buffer equal to the weight of the liver. The resulting homogenate was centrifuged at 800 G for 10 minutes at −1° C. The supernatant containing the broken liver cells minus nuclei and strands of connective tissue was called homogenate and was used as follows. For preparation of subcellular fractions, mitochondria were spun at 12,800 G for 10 minutes at −1° C in a Servall refrigerated centrifuge. Microsomes were then separated from the resulting supernatant by centrifuging at 144,000 G for 30 minutes at 0° C in a Spinco model L ultracentrifuge. The mitochondria and microsomes were washed once in ice-cold phosphate buffer, recentrifuged at 12,800 G for 10 minutes, and at 144,000 G for 30 minutes, respectively, and after discarding the wash, were resuspended in a volume of phosphate buffer equivalent to the volume of the homogenate from which they were obtained. The fraction remaining after removal of microsomes was called the supernatant. A portion of the original homogenate was boiled for 5 minutes at 100° C. It was then centrifuged at 144,000 G for 30 minutes. The resulting supernatant was removed and labeled KS fraction.

2. Incubation procedure. Homogenate and subcellular fractions were incubated in test tubes with BSP and other substrates at 37° C in a constant temperature water bath. The incubation volume varied from 1 to 3 ml (see figures and tables for details of different experiments). Incubation was terminated by addition of 0.36 ml saturated ammonium sulfate and 5 ml of absolute ethanol. After thorough mixing and standing for 20 minutes at room temperature, the tubes were centrifuged at 2,500 rpm for 10 minutes. On the average, 92.5 and 97.0 per cent of added BSP were recovered in the supernatants from tubes containing homogenate and subcellular fractions, respectively.

3. Chromatography and electrophoresis of BSP compounds. Descending chromatograms of the above supernatants were made on Whatman no. 1 filter-paper strips. BSP bands were identified by exposing the dried papers to ammonia vapors. The bands were eluted into 0.1 N KOH and the concentration of BSP in the eluate determined in a Beckman DU spectrophotometer set at 575 mμ. The chromatographic techniques and the methods of quantitating BSP on chromatograms have been described in detail in a previous publication (5). Electrophoresis of BSP compounds was carried out in a Spinco model R paper electrophoretic apparatus at 500 v for 3 hours, using 5 N acetic acid, pH 1.9, as the conducting solution. Inorganic bromide was detected in incubation mixtures by paper chromatography, using 80 per cent acetone as the solvent (8). The bromide spots

* This work was supported by research grants from the United States Public Health Service [H-3439], and the American Heart Association. Part of this work has appeared in abstract form (J. clin. Invest. 1960, **39**, 978) and was presented at the annual meeting of the American Association for the Study of Liver Diseases, Chicago, Ill., November, 1960.

† Established Investigator of the American Heart Association.

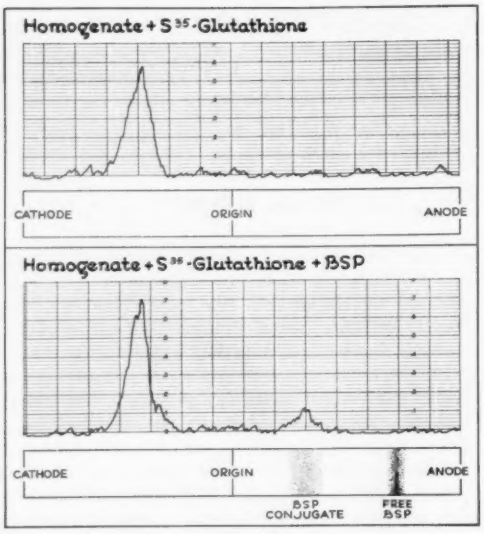


FIG. 1. INCORPORATION OF S^{35}-LABELED GLUTATHIONE INTO BSP CONJUGATE SYNTHESIZED BY LIVER HOMOGENATE *in vitro*. See text for details.

were developed by the technique of Mitchell (9). The concentration of bromide in bands from unknown solutions was quantitated visually, by comparison with the size and intensity of staining of bands of known NaBr standards applied on the same chromatogram. By applying varying aliquots of unknown solutions and several standards, it was possible to quantitate the bromide content with an accuracy of approximately ± 25 per cent.

4. Materials. Glutathione, S^{35}-labeled glutathione, oxidized glutathione and cysteine were purchased from Schwarz Laboratories. Cysteinyl glycine was purchased from Mann Research Laboratories. BSP and phenoltetrabromphthalein were obtained from Hynson, Westcott and Dunning.[1]

RESULTS

Incubation of BSP with a rat liver homogenate yielded two distinct BSP bands. One traveled with an Rf of 0.71 and chromatographically and electrophoretically was identical with free BSP. The other, with an Rf of 0.41, was chromatographically and electrophoretically identical with the major BSP conjugate found in rat bile, BSP-A, which in previous studies (5) was shown to be a conjugate of BSP and glutathione. Although identical chromatographic and electrophoretic mobilities suggest the same chemical structure, additional studies were carried out to determine the biochemical nature of the BSP compound formed *in vitro*. These revealed that the compound synthesized *in vitro* is the same as the major BSP compound formed *in vivo*. Conjugate was eluted from paper chromatograms and passed through a Dowex-50X, 200 to 400 mesh, ion exchange column (2) to remove any free amino acids or small peptides migrating with the same Rf as the BSP conjugate. The compound was then hydrolyzed with 5.9 N HCl in a sealed tube at 100° C for 18 hours. Two-dimensional amino acid chromatograms of the hydrolysate (10, 11) revealed glycine and glutamic acid in equimolar quantities, and relatively smaller amounts of alanine. In previous studies in which BSP conjugates were hydrolyzed in HCl, alanine was shown to represent cysteine minus its sulfhydryl group (5). The amino acids identified by paper chromatography are those contained in the tripeptide glutathione.

S^{35}-labeled reduced glutathione was incubated with a mixture of liver homogenate and BSP. After precipitation with ammonium sulfate and absolute ethanol, an aliquot of the supernatant was placed on paper and subjected to electrophoresis. BSP compounds were identified and the paper was then scanned for radioactivity[2] (Fig-

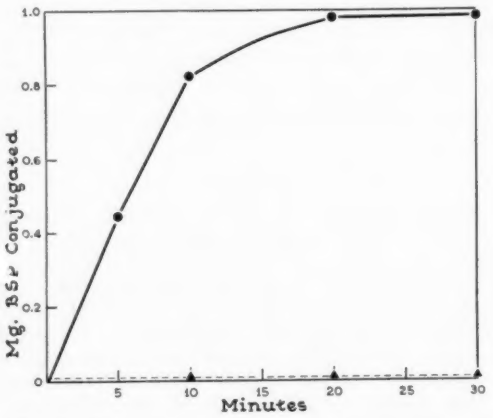


FIG. 2. FORMATION OF BSP CONJUGATE BY RAT LIVER HOMOGENATE. One ml of rat liver homogenate was incubated with 2 mg BSP at 37° C for varying times. Approximately 1 mg of BSP was conjugated by 20 minutes (●——●). Preheating liver homogenate at 100° C for 5 minutes destroyed its capacity to catalyze conjugate formation (▲- - - -▲).

[1] We are indebted to H. A. Brown Dunning, Jr., for a generous supply of phenoltetrabromphthalein.

[2] Model RSC-SA chromatogram scanner manufactured by Atomic Accessories, Inc.

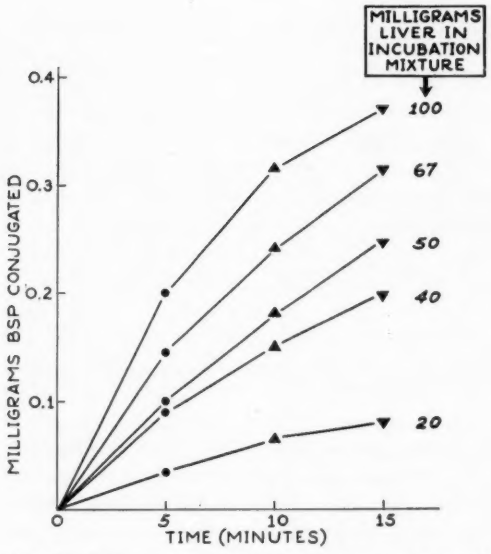


FIG. 3. BSP CONJUGATE FORMATION WITH VARYING AMOUNTS OF LIVER. Rat liver homogenate (500 mg liver, wet weight, per ml 0.1 M phosphate buffer, pH 7.8) was diluted with phosphate buffer to yield varying concentrations. Incubation mixture consisted of 1 ml diluted liver homogenate, 0.75 mg BSP in 0.2 ml phosphate buffer, and 4 mg glutathione in 0.1 ml phosphate buffer. Incubation was carried out in a constant temperature water bath set at 37° C. At varying time intervals, the reaction was stopped by addition of 0.36 ml saturated $(NH_4)_2SO_4$ and 5 ml absolute ethanol. Quantitation of BSP conjugate formation was carried out as described in Methods. A zero time reagent blank for each concentration of liver homogenate was set up as follows. One ml of liver homogenate was mixed with 4 mg glutathione in 0.1 ml phosphate buffer. The mixture was precipitated, as described above, by $(NH_4)_2SO_4$ and absolute ethanol. BSP, 0.75 mg in 0.2 ml phosphate buffer, was then added to the precipitated contents of the tube. The optical density of the eluted paper chromatographic area of the reagent blank, corresponding to the BSP conjugate area, was subtracted from the optical density of the eluted BSP conjugate to yield a net optical density due to BSP conjugate. At each time interval, larger amounts of BSP were conjugated with increasing amounts of liver in the incubation mixture.

ure 1). A peak of radioactivity conforming to glutathione and migrating toward the cathode was observed on the control paper prepared from a mixture of homogenate and S^{35}-labeled glutathione. In the homogenate mixture incubated with BSP, two BSP bands, identified as BSP conjugate and free BSP, had migrated toward the anode. The peak of radioactivity due to free glutathione was present on the cathode side, as in the control. Radioactivity was associated with the BSP band corresponding to BSP conjugate, indicating that radioactive glutathione was incorporated into this BSP compound.

The quantity of BSP conjugated during a 30-minute period in one experiment is shown in Figure 2. When the homogenate was heated for 5 minutes at 100° C, prior to incubation with BSP, its capacity to form the BSP conjugate was destroyed, suggesting that conjugate formation was catalyzed by an enzyme in liver. To strengthen this impression, varying amounts of liver homogenate made up to constant volume in 0.1 M phosphate buffer, pH 7.8, were incubated with quantities of BSP and glutathione shown to yield maximal amounts of conjugate formation. The system used is described in the legend of Figure 3. The rate of conjugate formation increased when increasing amounts of liver were added to the incubation mixture (Figures 3 and 4). The absolute quantity of BSP conjugated in each of three successive 5-minute intervals was greatest in the

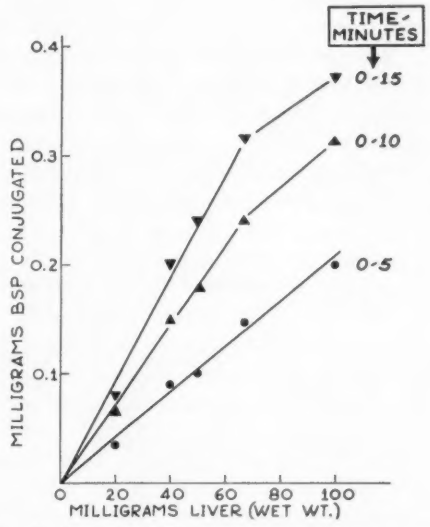


FIG. 4. RATE OF BSP CONJUGATE FORMATION AS FUNCTION OF QUANTITY OF LIVER IN INCUBATION MIXTURE. Data of Figure 3 were replotted. The total quantity of BSP conjugated in the intervals 0 to 5, 0 to 10, and 0 to 15 minutes was plotted against the amount of liver in incubation mixture. The rate of conjugate formation was proportional to the quantity of liver present, except for the 0 to 10 and 0 to 15 minute values of the mixture containing 100 mg of liver. See text for details.

TABLE I

Incubation of BSP with homogenate and subcellular fractions of rat liver *

	Homogenate	Homogenate heated at 100° C for 5 min	Supernatant	Mitochondria	Microsomes	Mitochondria + KS	Microsomes + KS	Supernatant + mitochondria	Supernatant + microsomes	Microsomes + mitochondria	Microsomes + mitochondria + KS
BSP conjugate											
Rf	0.41		0.45					0.42	0.42		
Quantity BSP conjugated mg/5 min	0.448	0	0.452	0	0	0	0	0.466	0.410	0	0
Free BSP											
Rf	0.71	0.72	0.71	0.73	0.72	0.72	0.70	0.72	0.71	0.74	0.72

* Incubation mixture contained 1 ml of each specified fraction with final volume brought to 3 ml by addition of 0.1 M phosphate buffer, pH 7.8. Each tube was warmed in a 37° C water bath for 5 minutes. Then, 2 mg of BSP was added to each tube and incubation continued for 5 minutes, after which it was terminated as described in Methods.

first 5-minute period and least in the last. Nevertheless, at each time interval, the amount of conjugate formed was proportional to the quantity of liver present, and thus presumably to enzyme concentration. Deviation from proportionality was observed only in periods 0 to 10 minutes and 0 to 15 minutes, with the incubation mixture containing the largest quantity of liver, namely 100 mg. This deviation would seem to be explained best by a lack of sufficient substrate in the incubation mixture to saturate fully the enzyme contained in 100 mg of liver after 5 minutes of incubation.

In order to localize the site of enzyme activity within the cell, BSP was incubated with various subcellular fractions of liver. BSP conjugate was formed only in those tubes containing the supernatant fraction of liver (Table I). Furthermore, all of the conjugating activity of the liver homogenate was accounted for in the supernatant fraction. No conjugate was formed with mitochondria, microsomes, or mixture of these, even with the addition of KS fraction. Addition of mitochondria or microsomes to the supernatant did not enhance its conjugating activity.

Supernatant fraction of liver which had been dialyzed against phosphate buffer for 24 hours at 0° C did not form conjugate when incubated with BSP. Conjugating activity was restored, however, upon the addition of KS fraction. Furthermore, BSP conjugate was also synthesized when glutathione *alone* was added to dialyzed supernatant. No additional cofactor was required.

TABLE II

Quantity of BSP conjugated with glutathione, cysteinyl glycine and cysteine *

Species	Quantity of liver in 1 ml	BSP conjugated in 15 minutes with: No peptide or amino acid added	Glutathione (4 mg)	Cysteinyl glycine (2.3 mg)	Cysteine (1.6 mg)
	mg	*mg*		*mg*	
Human†	40	0	0.447	0.068	0.015
Rat	40	0	0.248	0.039	0.013
	40	0	0.211	0.030	0.017
	40	0	0.162	0.021	0.016
	40	0	0.224	0.027	0.009
Dog	100	0	0.069	0.007	0.013
Mouse	40	0	0.158	0.015	0.016
Guinea pig	40	0	0.149	0.017	0.012
Cat	200	0	0.053		

* Incubation mixture consisted of 1 ml of diluted liver homogenate contained in 0.1 M phosphate buffer, pH 7.8; 0.75 mg BSP in 0.2 ml phosphate buffer; and peptide or amino acid in 0.1 ml phosphate buffer. Tubes were incubated for 15 minutes at 37° C.

† Liver biopsy obtained at laparotomy for cholelithiasis.

The quantity of BSP conjugated by liver homogenate upon incubation with several peptides and amino acids was examined. Human, rat, dog, mouse, guinea pig and cat liver homogenates diluted with phosphate buffer did not form conjugate with BSP (Table II). BSP conjugates were formed, however, when glutathione, cysteinyl glycine or cysteine was added to the incubation mixture. Much more conjugate was formed per unit time with glutathione than with equimolar quantities of cysteinyl glycine or cysteine. No conjugate was formed with oxidized glutathione, cystine, taurine, methionine, glycine, glutamic acid or alanine.

Chromatographs of the BSP compounds synthesized by a 40 to 60 per cent ammonium sulfate fraction of the soluble supernatant fraction of rat liver were sprayed with alcoholic KOH (1 part 2 N KOH and 19 parts absolute ethanol). The BSP bands were identified by their purple color and, when examined with ultraviolet light, by their red fluorescence. Although BSP bands corresponding only to BSP-A and BSP-D were seen in room light, four BSP bands were identified in the mixture containing glutathione by means of ultraviolet light. Three bands were identified when BSP was incubated with cysteinyl glycine. Only two bands were seen with cysteine (Figure 5). For comparison, a chromatogram of BSP compounds excreted in the bile of a rat after BSP was administered intravenously is shown. Four BSP bands are identified. BSP-A, B and C represent BSP conjugates, whereas BSP-D is free BSP. It is apparent that the bands in the glutathione mixture conform to the bands in BSP bile. Band A is the most prominent conjugate. Only traces of B and C can be seen. Cysteinyl glycine and BSP yielded bands corresponding to BSP conjugates B and C (12). In the cysteine mixture, only one conjugate was seen and this conformed to BSP-B. A mixture of the compounds synthesized from glutathione, cysteinyl glycine and cysteine yielded a chromatograph resembling BSP bile very closely.

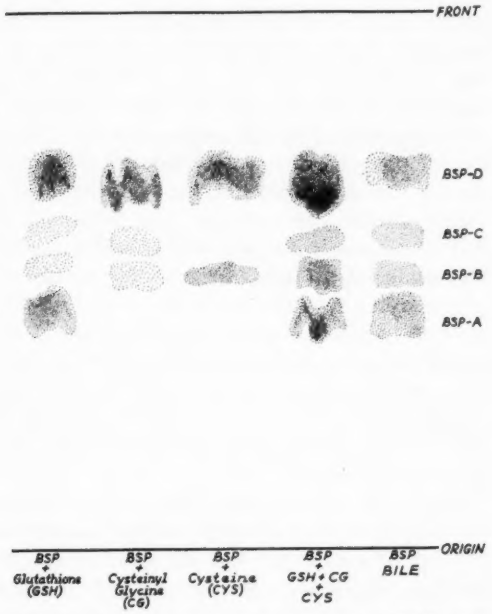


FIG. 5. DRAWING OF CHROMATOGRAMS OF BSP COMPOUNDS IDENTIFIED IN INCUBATION MIXTURES CONTAINING LIVER ENZYME, BSP AND VARIOUS PEPTIDES AND AMINO ACIDS. The liver enzyme was a 40 to 60 per cent $(NH_4)_2SO_4$ fraction of the soluble supernatant fraction of the liver. BSP and glutathione yielded four bands. BSP-A and BSP-D were prominent. Only traces of BSP-B and BSP-C were identified. BSP and cysteinyl glycine yielded three bands, BSP-B, BSP-C and BSP-D. BSP and cysteine yielded only BSP-B and BSP-D. Mixing the contents of tubes originally containing BSP and glutathione, BSP and cysteinyl glycine, and BSP and cysteine yielded a chromatogram resembling that of bile containing excreted BSP very closely.

TABLE III

Bromide released during glutathione conjugation

Compound	Enzyme*	Glutathione	Cpd. conjugated	Moles bromide released/mole conjugated†
mg	*ml*	*mg*	*mg/30 min*	
BSP				
2		4	0.15	1
2	1	4	1.61	1
2	1 (Δ 100° C 10 min)	4	0.06	1
2	1		0	0
	1	4	0	0
PTBP‡				
1.5	1	4	0.17	1
PP§				
0.75	1	4	0	0

* 40 to 60 per cent $(NH_4)_2SO_4$ fraction of liver supernatant fraction.
† The figure 1 is an approximation of actual ratio which could have varied between 0.75 and 1.25 because of possible errors in quantitating bromide (see Methods).
‡ Phenoltetrabromphthalein dissolved in 0.8 ml of 66.7% ethyl alcohol.
§ Phenolphthalein dissolved in 0.8 ml of 66.7 per cent ethyl alcohol.

Inorganic bromide was released during conjugation of BSP and glutathione (Table III). In-

TABLE IV

BSP-conjugating activity of various organs

Tissue	BSP conjugated
	mg/g wet wt/5 min
Liver	2.300
Kidney	0.108
	0.058
Spleen	0.153
Intestine	0.015
Lung	0.058
Brain	0.230
	0.147
Skeletal muscle*	0.010
Red blood cells	0

* Difficult to homogenize.

cubation of 2 mg of BSP with 4 mg of glutathione for 30 minutes yielded 0.15 mg of conjugated BSP. When enzyme was added to the incubation mixture, 1.61 mg of BSP was conjugated. Enzyme activity was destroyed by heating at 100° C for 10 minutes. No conjugate was formed when either BSP or glutathione was omitted from the incubation mixture. In the studies in which BSP conjugation occurred, approximately 1 mole of bromide ion was released for each mole of conjugate formed. Free bromide ion was not detected in the absence of conjugate formation, suggesting that bromide release is intimately related to BSP conjugation. This was supported by results obtained upon incubation of compounds related to BSP with enzyme and glutathione. When phenoltetrabromphthalein (PTBP, BSP minus the 2 sulfonic acid groups), a halogen-containing compound, was the substrate, a small amount of conjugate was formed with release of approximately 1 mole of bromide for each mole of conjugate formed. Phenolphthalein (PP, BSP minus the 2 sulfonic acid groups and 4 bromine atoms), a halogen-free compound, did not form a glutathione conjugate.

The distribution of enzyme activity in various tissues of the rat other than liver was examined. Small amounts of enzyme activity were found in most tissues (Table IV). None was detectable in red blood cells.

DISCUSSION

A liver enzyme that catalyzes the conjugation of BSP with glutathione has been demonstrated in the present studies. Although a small quantity of BSP-glutathione conjugate is formed per unit time when BSP and glutathione are incubated *in vitro*, the rate of conjugation is enhanced considerably upon addition of liver homogenate to the incubation mixture. As expected in reactions catalyzed enzymatically, the rate of BSP conjugate formation is proportional to the amount of liver homogenate added to the incubation mixture, and thus presumably to enzyme concentration. Furthermore, enzyme activity is destroyed by heating liver homogenate at 100° C.

Glutathione is the optimal substrate for conjugation with BSP in the enzyme-catalyzed reaction *in vitro*. Cysteine and cysteinyl glycine also combine with BSP, but the rate at which these compounds form BSP conjugates is much slower than with glutathione. These observations support previous conclusions that conjugation of BSP with glutathione is the major pathway of BSP metabolism *in vivo* (5, 6). A free sulfhydryl group appears to be necessary for conjugation, since oxidized glutathione, cystine and methionine do not form BSP conjugates. BSP conjugation also did not occur with glycine, glutamic acid and alanine —amino acids identified after acid hydrolysis of BSP conjugates (2–6)—a finding which further supports the conclusion that it is glutathione that is conjugated with BSP. It is of interest that most of the glutathione in liver is contained in the soluble supernatant fraction (13). All of the BSP-conjugating enzyme activity is also found in this compartment of liver cells. Such localization of enzyme and substrate is optimal for BSP-glutathione conjugation.

The release of approximately 1 mole of bromide ion from BSP for each mole of BSP-glutathione conjugate formed was regularly observed in both the uncatalyzed and enzyme-catalyzed reactions in the present studies. The release of inorganic bromide during the uncatalyzed reaction has also been reported recently by Javitt and his associates (6). These observations are at variance with the findings of Krebs and Brauer (1), which indicated that the ratio of bromine to sulfur in the major BSP metabolite in rat bile was the same as that of standard BSP, namely 4 bromine to 2 sulfur atoms. Since the major BSP conjugate in rat bile, BSP-A, appears to be composed of 1 mole of BSP conjugated with 1 mole of glutathione (5), one would expect this compound to

have a ratio of 3 bromine to 3 sulfur atoms. A similar ratio of bromine to sulfur, 3:3, should also exist in the other BSP metabolites found in rat bile, BSP-B and BSP-C. Javitt and coworkers have also reported the presence of a BSP-diglutathione compound after incubation of BSP and glutathione *in vitro,* and have suggested that this compound appears occasionally in small quantities in rat bile (6). Although we have never identified such a compound in rat bile, it would be expected to have a bromine to sulfur ratio of *2*:4.

Several observations indicate that bromide release is intimately related to BSP conjugation with glutathione. Bromide is released whenever BSP-glutathione is formed, whether the reaction is enzyme-catalyzed or not. It is not released in the absence of conjugate formation. Phenoltetrabromphthalein, a bromine-containing compound closely related structurally to BSP, forms a glutathione conjugate with release of inorganic bromide. However, phenolphthalein, a halogen-free compound, does not form a glutathione conjugate. Conjugation of BSP with glutathione appears to be dependent, therefore, on the presence of its halogen groups. Previous studies indicated that glutathione was conjugated with BSP in thioether linkage (5, 6). The available sites for conjugation on the BSP molecule included the free positions on the phenolic groups and the positions occupied by the 4 bromine atoms. The present data suggest that the sulfur group of glutathione attaches to BSP at the site of bromine removal. The observations that inorganic bromide is released from BSP upon incubation of BSP and glutathione in the absence of enzyme, and that no bromide is released when BSP is incubated with enzyme in the absence of glutathione, suggest that a function of the enzyme is to enhance the reactivity of the sulfhydryl group of glutathione.

In a previous publication (5), it was concluded that BSP-A, the major BSP compound in rat bile, is a conjugate of BSP and glutathione. BSP and BSP-C, minor BSP bands, were felt to contain isomers of BSP-glutathione as well as mixtures of its hydrolytic products, such as BSP-cysteinyl glycine and BSP-cysteine. These conclusions are supported by the current studies in which BSP compounds were formed enzymatically *in vitro.* Isomers of BSP-glutathione would be explained by conjugation of glutathione at different sites of bromine removal.

BSP-conjugating enzyme has been identified in the livers of many species. It has also been found to a lesser extent in many other organs. These latter findings are consistent with the observations of Rosenau, Carbone and Grodsky, indicating that BSP metabolites may appear in blood of hepatectomized animals (14).

The precise role of BSP conjugation in the movement of BSP from blood to bile is as yet unknown. Although it is recognized that other factors, such as hepatic blood flow and protein binding of BSP in plasma, may play a role in the hepatic uptake of BSP, the demonstration that BSP metabolism depends on glutathione and a liver enzyme suggests that the impaired removal of BSP from blood, in liver disease, may be related also to either alterations in hepatic glutathione content or conjugating enzyme activity, or both.

SUMMARY

An enzyme that catalyzes the conjugation of BSP and glutathione has been identified in the soluble supernatant fraction of liver. Glutathione is the optimal substrate. No requirement for cofactor has been demonstrated. Inorganic bromide is released from BSP during conjugation. The sulfhydryl group of glutathione appears to conjugate with BSP at the site of bromine removal.

ACKNOWLEDGMENT

The authors are indebted to Mrs. Ruthie Rose and to Mr. Allen White for valuable technical assistance.

REFERENCES

1. Krebs, J. S., and Brauer, R. W. Metabolism of sulfobromophthalein sodium (BSP) in the rat. Amer. J. Physiol. 1958, **194**, 37.
2. Combes, B. The biliary excretion of sulfobromophthalein sodium (BSP) in the rat as a conjugate of glycine and glutamic acid. J. clin. Invest. 1959, **38**, 1426.
3. Meltzer, J. I., Wheeler, H. O., and Cranston, W. I. Metabolism of sulfobromophthalein sodium (BSP) in dog and man. Proc. Soc. exp. Biol. (N. Y.) 1959, **100**, 174.
4. Grodsky, G. M., Carbone, J. V., and Fanska, R. Identification of metabolites of sulfobromophthalein. J. clin. Invest. 1959, **38**, 1981.
5. Combes, B., and Stakelum, G. S. Conjugation of sulfobromophthalein sodium with glutathione in

thioether linkage by the rat. J. clin. Invest. 1960, **39**, 1214.

6. Javitt, N. B., Wheeler, H. O., Baker, K. J., Ramos, O. L., and Bradley, S. E. The intrahepatic conjugation of sulfobromophthalein and glutathione in the dog. J. clin. Invest. 1960, **39**, 1570.
7. Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S., and Cottone, M. A. A method for isolating intact mitochondria and nuclei from the same homogenate, and the influence of mitochondrial destruction on the properties of cell nuclei. J. biophys. biochem. Cytol. 1955, **1**, 139.
8. Pollard, F. H., and McOmie, J. F. W. Chromatographic Methods of Inorganic Analysis, With Special Reference to Paper Chromatography. New York, Academic Press, 1953.
9. Mitchell, L. C. Separation and identification of acids by paper chromatography. I. The halides. J. Ass. off. agr. Chem. 1955, **38**, 832.
10. Dent, C. E. A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-"collidine" filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. Biochem. J. 1949, **43**, 169.
11. Datta, S. P., Dent, C. E., and Harris, H. An apparatus for the simultaneous production of many two-dimensional paper chromatograms. Science 1950, **112**, 621.
12. Gutcho, M., and Laufer, L. Paper chromatography of glutathione and its hydrolysis products *in* Glutathione. New York, Academic Press, 1954, p. 79.
13. Edwards, S., and Westerfeld, W. W. Blood and liver glutathione during protein deprivation. Proc. Soc. exp. Biol. (N. Y.) 1952, **79**, 57.
14. Rosenau, W., Carbone, J. V., and Grodsky, G. M. Metabolism of sulfobromophthalein in hepatectomized and hepatectomized-nephrectomized dog. Proc. Soc. exp. Biol. (N. Y.) 1959, **102**, 131.

LEUKOKINETIC STUDIES. IV. THE TOTAL BLOOD, CIRCULATING AND MARGINAL GRANULOCYTE POOLS AND THE GRANULOCYTE TURNOVER RATE IN NORMAL SUBJECTS *

By J. W. ATHENS, O. P. HAAB,† S. O. RAAB, A. M. MAUER,‡ H. ASHENBRUCKER, G. E. CARTWRIGHT AND M. M. WINTROBE

(From the Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

(Submitted for publication January 19, 1961; accepted February 17, 1961)

When granulocytes are labeled with diisopropylfluorophosphate (DFP^{32}) and then returned to the circulation of the donor, the labeled granulocytes are distributed in a pool of cells which is approximately two times larger than that calculated from the blood volume and the concentration of granulocytes in the circulating venous blood (1, 2). This pool has been referred to as the total blood granulocyte pool (TBGP) and it consists of two subcompartments or pools. These pools have been designated the circulating granulocyte pool (CGP) and the marginal granulocyte pool (MGP). The size of the CGP can be calculated from the blood volume and the absolute granulocyte count. Equilibration between the granulocytes in the CGP and in the "noncirculating" or MGP is sufficiently rapid and complete to allow these two pools to be considered as one kinetically, and the size of the TBGP can be determined by the isotope dilution principle. Since the cells are removed from the TBGP in an exponential fashion with a mean half-time disappearance ($T_{\frac{1}{2}}$) of 6.6 hours, the granulocyte turnover rate (GTR), that is, the number of granulocytes turned over through the blood in a unit of time, can be calculated.

The purpose of this paper is to present data on the GTR in normal subjects, as well as additional data on the size of the TBGP, CGP and MGP in normal subjects. The influence of steroids, physical exercise, epinephrine and bacterial endotoxin on these parameters in normal subjects has also been studied.

* This investigation was supported by a research grant (A-4489) and a graduate training grant (2A-5098) from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

† Fellow of the Leukemia Society, Inc., New York, N. Y.

‡ American Cancer Society Postdoctoral Fellow.

Several preliminary reports of this work have been published (3, 4).

MATERIALS AND METHODS

Twenty-five volunteers from the Utah State Prison, in addition to the 45 subjects previously reported (1, 2), were used in these studies. All of the subjects were normal healthy males, 20 to 50 years of age, with normal leukocyte values. The subjects ate breakfast at 6:00 a.m. and the infusion of labeled blood was begun between 8:00 and 10:00 a.m.

The methods for labeling blood *in vitro*, for the isolation of leukocytes from the blood samples, for the determination of leukocyte radioactivity, and for the calculation of the TBGP, CGP and $T_{\frac{1}{2}}$ have been described in previous publications (1, 2, 5).

The GTR, defined as the number of granulocytes turned over through the blood each day per kilogram body weight, was calculated from the following equation (6):

$$GTR = 24 \times 0.693/T_{\frac{1}{2}}\ (hrs) \times TBGP\ (no.\ cells \times 10^7/kg).$$

The DFP^{32} was purchased from the New England Nuclear Corp., Boston, Mass., as a dilute solution (1.0 mg per ml) in sterile, anhydrous propylene glycol. The concentration of DFP was determined in our own laboratory by a modification of the method of Marsh and Neale (7). The specific activity of the material at the time it was used was 74 to 224 μc per mg. Except when noted otherwise, DFP^{32} was added to the blood (400 to 500 ml) in the bag in a concentration of 0.3 μg per ml of whole blood.

RESULTS

TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR in normal subjects. The values for the total blood granulocyte pool, circulating granulocyte pool, marginal granulocyte pool, half-time disappearance and granulocyte turnover rate in normal male subjects are presented in Table I. The pool sizes were determined on a total of 70 normal subjects, including the 45 subjects previously reported (2). The $T_{\frac{1}{2}}$ and GTR were determined on 45 of the 70 individuals.

TABLE I

The total blood granulocyte pool (TBGP), circulating granulocyte pool (CGP), marginal granulocyte pool (MGP), half-time disappearance ($T_{\frac{1}{2}}$) and granulocyte turnover rate (GTR) in normal male subjects

Determination	Mean	±1 SD*	Determined range
TBGP × 10^7/kg	65.0	22.4	33–117
CGP × 10^7/kg	31.7	11.1	15–54
MGP × 10^7/kg	33.3	16.0	9–79
$T_{\frac{1}{2}}$ (hrs)	6.6	1.16	4–9
GTR × 10^7/kg/day	179.9	74.3	86–341

* Standard deviation.

To determine the reproducibility of these parameters in normal subjects, studies were carried out in four subjects and repeated in the same subjects 1 month later. The concentration of DFP^{32} used was 0.15 μg per ml of whole blood rather than the customary 0.30 μg per ml so that the amount of radiation which each subject received was not increased. The results are presented in Table II.

TABLE II

The reproducibility of the measurement of the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR *

Determination	Subject no.	First measurement	Second measurement
TBGP × 10^7/kg	IV–30	91	59
	IV–28	57	106
	IV–34	66	51
	IV–32	38	53
	Mean	63	67
CGP × 10^7/kg	IV–30	45	38
	IV–28	29	33
	IV–34	26	25
	IV–32	15	19
	Mean	29	29
MGP × 10^7/kg	IV–30	46	21
	IV–28	28	73
	IV–34	40	26
	IV–32	23	34
	Mean	34	38
$T_{\frac{1}{2}}$ (hrs)	IV–30	6.5	8.5
	IV–28	6.6	6.8
	IV–34	6.4	5.0
	IV–32	7.4	5.5
	Mean	6.7	6.5
GTR × 10^7/kg/day	IV–30	231	115
	IV–28	145	259
	IV–34	170	168
	IV–32	86	160
	Mean	158	175

* See Table I for abbreviations.

Influence of prednisone on the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR. Each of five subjects was given an infusion of autogenous, DFP^{32}-labeled blood (0.15 μg of DFP^{32} per ml). Control values for the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR were obtained on each of the subjects. The subjects were then given 40 mg of prednisone daily by the oral route for 11 days. The absolute granulocyte count increased an average of 75 per cent (35 to 120) by the eighth day and remained constant over the next 3 days. Measurements were repeated on the eighth day of steroid administration. The results are presented in Table III.

TABLE III

Influence of prednisone on the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR *

Determination	Before prednisone	After prednisone
TBGP × 10^7/kg	48 (37–55)	80 (54–112)
CGP × 10^7/kg	29 (18–45)	51 (36–69)
MGP × 10^7/kg	19 (10–32)	29 (18–43)
$T_{\frac{1}{2}}$ (hrs)	6.8 (6.0–8.4)	10.3 (9.0–13.1)
GTR × 10^7/kg/day	119 (85–142)	127 (100–153)

* The values represent the means of the determinations in 5 subjects. The figures in parentheses refer to range. See Table I for abbreviations.

Prednisone increased the CGP an average of 76 per cent, the MGP 53 per cent and the TBGP 67 per cent. In spite of this increase, the GTR was not altered significantly because of a prolongation of the $T_{\frac{1}{2}}$.

Influence of exercise on the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR. In a previous study (2) it was demonstrated that physical exercise resulted in a shift of granulocytes from the MGP to the CGP without an increase in the TBGP.

To determine if exercise influences the rate of disappearance of granulocytes from the blood, six subjects were given an infusion of autogenous, DFP^{32}-labeled blood. The pool sizes were determined and 6 hours later, at which time the slope of the disappearance curve was well established, the subjects ran 440 yards. In none of the sub-

jects was the slope of the disappearance curve altered following exercise, in spite of the increase in the concentration of granulocytes in the CGP. The disappearance curve in a representative subject is presented in Figure 1.

Since exercise did not influence the slope of the disappearance curve, the GTR was calculated for each of the subjects. The data are summarized in Table IV. The values for the $T_{\frac{1}{2}}$ and for the GTR were within normal limits.

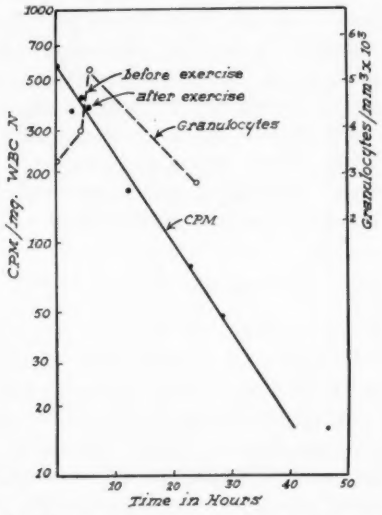


FIG. 1. INFLUENCE OF EXERCISE ON THE GRANULOCYTE DISAPPEARANCE CURVE AND ON THE CONCENTRATION OF GRANULOCYTES IN THE CIRCULATION. Six hours after the infusion of labeled blood, the subject ran 440 yards. The specific activity (cpm/mg WBC N) was the same immediately after exercise as before exercise, in spite of the increase in the granulocyte count. Thus, the cells which were mobilized into the CGP had the same specific activity as those already present in this pool. Furthermore, this shift of cells into the pool had no influence on the slope of the disappearance curve.

Influence of epinephrine on the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR. In the previous publication (2), evidence was presented that epinephrine, like physical exercise, results in a shift of granulocytes from the MGP to the CGP, the TBGP remaining unchanged.

To determine if epinephrine influences the rate of disappearance of granulocytes from the blood, four subjects were given an infusion of autogenous, DFP^{32}-labeled blood to which 0.5 mg of epinephrine was added. The pool sizes and the $T_{\frac{1}{2}}$ were then determined. The $T_{\frac{1}{2}}$ and the GTR were within the normal limits in all four subjects (Table IV).

TABLE IV

Influence of exercise and of epinephrine on the TBGP, CGP, MGP, $T_{\frac{1}{2}}$ and GTR *

Determination	Exercise†	Epinephrine‡
TBGP × 10^7/kg	53 (40–85)	55 (24–91)
CGP × 10^7/kg	46 (35–52)	35 (18–40)
MGP × 10^7/kg	7 (0–33)	20 (6–52)
$T_{\frac{1}{2}}$ (hrs)	6.7 (5.5–7.6)	5.8 (4.6–7.6)
GTR × 10^7/kg/day	132 (102–214)	185 (86–277)

* See Table I for abbreviations.
† Mean values for 6 subjects.
‡ Mean values for 4 subjects. The figures in parentheses refer to range.

Influence of bacterial endotoxin on the TBGP, CGP and MGP. The administration of bacterial endotoxin in small amounts to human subjects is associated with a granulocytosis 5 to 10 hours after the substance is given intravenously (8). A transient, mild granulocytopenia has been observed in some but not in all subjects 1 to 2 hours after the intravenous injection of endotoxin.

The influence of endotoxin (highly purified protein-free lipopolysaccharide from *Salmonella abortus equi*) on the TBGP, CGP and MGP was determined in six subjects. The pool sizes were measured on three separate occasions; before the intravenous administration of 0.1 μg of bacterial

TABLE V

Influence of bacterial endotoxin on the TBGP, CGP and MGP *

Determination	Before endotoxin	1.5 Hrs after endotoxin		5.0 Hrs after endotoxin
No. of subjects	6	2	4	6
TBGP × 10^7/kg	63 (36–115)	69 (49–89)	108 (88–126)	134 (111–146)
CGP × 10^7/kg	29 (21–36)	20 (13–28)	36 (32–41)	64 (51–95)
MGP × 10^7/kg	34 (9–79)	49 (36–61)	72 (56–85)	70 (34–86)
CGP/MGP	0.86	0.41	0.50	0.91

* The figures in parentheses refer to range. See Table I for abbreviations.

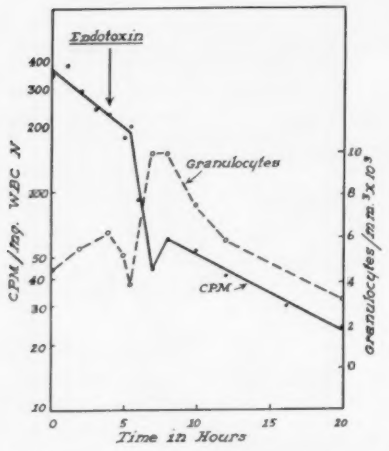


FIG. 2. INFLUENCE OF BACTERIAL ENDOTOXIN ON THE GRANULOCYTE DISAPPEARANCE CURVE AND ON THE CONCENTRATION OF GRANULOCYTES IN THE CIRCULATION. Bacterial endotoxin, 0.1 μg, was injected intravenously 4 hours after the infusion of labeled blood. A mild, transient granulocytopenia occurred, reaching a maximum 1.5 hours after the endotoxin was given. During the granulocytopenic phase the specific activity (cpm/mg WBC N) did not change. As the concentration of granulocytes in the CGP increased, the specific activity of the granulocytes decreased sharply, indicating that unlabeled cells were entering the pool. Thereafter, the slope of the disappearance curve was altered as compared with the slope of the curve prior to the administration of endotoxin.

endotoxin and 1.5 and 5.0 hours after endotoxin was given. On each occasion the blood was labeled with 0.1 μg of DFP^{32} per ml of whole blood. The results are presented in Table V.

A transient granulocytopenia was detected in two of the six subjects 1.5 hours after endotoxin was given. In these two subjects there was a shift of granulocytes from the CGP to the MGP, without an increase in the TBGP at this time. In the other four subjects, there was an appreciable increase in the TBGP 1.5 hours after endotoxin. This increase was due primarily to an increase in MGP.

Five hours after endotoxin administration, both the CGP and the MGP were greatly increased but the ratio of the size of the CGP to the MGP was unaltered as compared with the values prior to the administration of endotoxin.

The influence of endotoxin on the granulocyte disappearance curve was studied in three subjects. Four hours after an infusion of autogenous, DFP^{32}-labeled blood, at which time the slope of the disappearance curve was established, each subject was given 0.1 μg of endotoxin intravenously. The results in one subject with a transient early granulocytopenia are presented in Figure 2.

During the transient granulocytopenic phase which appeared 1.5 hours after endotoxin administration, the rate of decrease in the specific activity of the granulocytes in the circulation was unaltered. This would be expected since removal of granulocytes from the CGP would not alter the specific activity of the cells remaining. On the other hand, during the phase when the granulocyte count was increasing, there was a precipitous decline in the specific activity of the granulocytes in the CGP. Thus, during this period unlabeled granulocytes were entering this compartment diluting the specific activity of the cells.

DISCUSSION

In the physiological steady state, the GTR is equivalent to the rate of production of granulocytes by the bone marrow and the rate of utilization of granulocytes by the tissues. The study of production and destruction rates in patients with disorders of granulopoiesis should be of considerable interest and should add to our understanding of the pathophysiology of these diseases, just as a study of red cell production and destruction rates has added to our understanding of the pathophysiology of the anemias.

Direct quantitative measurement of the GTR has not been possible heretofore. Our studies indicate that normal subjects produce and utilize (destroy) 180×10^7 granulocytes per day per kg of body weight. This value is of the same order of magnitude as the value of 123×10^7 cells which Killmann, Cronkite, Fliedner and Bond (9) calculated from the observed ratio between mitoses in the erythroid and myeloid series, assuming an equal mitotic time for the two series.

The range of values for the GTR which we have obtained in normal subjects is wide. This probably is due in part to cumulative errors in the methods and in part to physiological variations. The GTR was reproducible in a given subject within a factor of two. The variation observed when the measurements were repeated in the

same subject 1 month later could have been due to inaccuracies of the method, to physiological variations, or to both. Since it is well known that the leukocyte count varies in a subject from hour to hour and from day to day, it would be expected that the GTR would vary to a greater degree than the erythrocyte turnover rate. Our experiments do indicate that the variation in the GTR in a given subject from time to time is nearly as great as the differences which are observed beween individuals. Comparisons between the mean value of at least four determinations would seem to be required before conclusions can be drawn reliably. The difference between the mean values for the GTR repeated in four subjects was 10 per cent.

In addition to the possible sources of error in the estimation of the blood pools and $T_{\frac{1}{2}}$ which were discussed previously (1, 2, 5), the assumption is made in the calculation of the GTR that granulocytes do not return from the tissues to the TBGP. Several observers (10, 11) have assumed that there is an appreciable exchange of granulocytes between blood and tissues. However, experimental support for the existence of this exchange was not obtained by Craddock, Perry, Ventzke and Lawrence (8) or by Patt, Maloney and Jackson (12). From our own studies an appreciable return from tissues would seem unlikely, since the curve of disappearance of labeled cells from blood is a single exponential which decreases to background counting rates (1). If labeled granulocytes returned from the tissues to the blood, it would be expected that an equilibrium level would be approached. However, it should be pointed out that failure to observe such an equilibrium does not exclude the possibility that there is some exchange of granulocytes between these two pools. If the size of the tissue pool is sufficiently large by comparison with the TBGP, then a small return of granulocytes to the circulation may not have been detected. The lower limit of the size of a tissue pool of granulocytes which could exist and be compatible with our observations has been defined elsewhere (13).

In the present investigation perturbations were induced in the system with physical exercise, epinephrine, prednisone and bacterial endotoxin (Figure 3). From these studies certain characteristics of the system can be defined.

Exercise and epinephrine, as reported previously (2), do not alter the size of the TBGP but cause a shift in cells from the MGP to the CGP. This shift of cells has no influence on the rate of disappearance of cells from the TBGP or on the GTR. This is further evidence that these two blood pools can be considered as one pool kinetically. Prednisone, on the other hand, increased the size of both the CGP and the MGP. The $T_{\frac{1}{2}}$ was prolonged and the GTR was unaltered. Whether the TBGP was increased by mobilizing cells from the bone marrow or by prolonging the rate of removal of cells from the TBGP is not apparent from our studies. It can be stated that the rate of production and utilization of granulocytes at the new equilibrium level was not increased since the GTR was unaltered.

The alterations following administration of endotoxin differed from those with prednisone, physical exercise and epinephrine. Endotoxin produced two effects, namely, a shift of granulocytes from the CGP into the MGP and an influx of cells into the TBGP. In two of the six subjects margination preceded the influx of cells into the TBGP, and a transient neutropenia, that is, decrease in the size of the CGP, occurred. In the

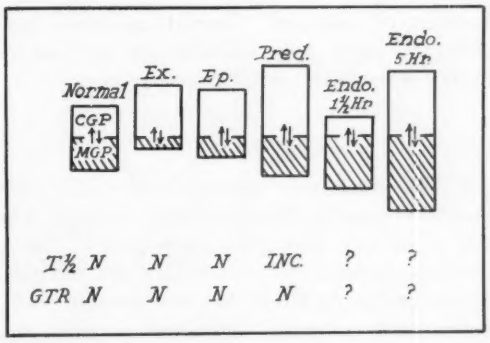


FIG. 3. SCHEMATIC SUMMARY OF THE INFLUENCE OF EXERCISE (EX.), EPINEPHRINE (EP.), PREDNISONE (PRED.) AND BACTERIAL ENDOTOXIN (ENDO.) ON THE CIRCULATING GRANULOCYTE POOL, MARGINAL GRANULOCYTE POOL, HALF-TIME DISAPPEARANCE RATE, (IN HOURS), AND GRANULOCYTE TURNOVER RATE $\times 10^7$ CELLS/KG/DAY. The total areas of the rectangles represent the relative sizes of the TBGP. The CGP is represented by the open area, the MGP by the hatched area. The relative values for the pool sizes for the two subjects with neutropenia 1.5 hours after the administration of endotoxin are given and are compared with those of all subjects 5.0 hours after endotoxin administration (N = normal, Inc. = increased.)

other four subjects margination and mobilization of cells into the TBGP occurred simultaneously. Although the ratio of the CGP to MGP was decreased, there was no reduction in the size of the CGP and, therefore, no neutropenia. Five hours after endotoxin administration, the CGP as well as the MGP was greatly increased in all six subjects. Since granulocytes presumably do not move from the tissues to the blood, the unlabeled cells which entered the TBGP must have been released from the bone marrow pool of granulocytes. Craddock and co-workers (8) have presented other evidence that this is the case and they have suggested that the administration of endotoxin may be a useful procedure in assessing marrow granulocyte reserve. It would be interesting to know if endotoxin increased the GTR. Because the size of the TBGP was constantly changing after endotoxin was given, the system was not in a steady state and this calculation could not be considered accurate under these conditions.

The GTR is probably influenced by a wide variety of physiological and pathological factors. It is to be regretted that our studies in normal subjects have been limited to males. We have not felt justified in exposing normal females in the childbearing period of life to even these small amounts of radiation. Diurnal variations, psychological stress, variation with age, and the influence of meals on the GTR deserve study.

SUMMARY

1. The granulocyte turnover rate (GTR) has been determined in 45 normal male subjects. The size of the total blood granulocyte pool (TBGP), circulating granulocyte pool (CGP) and marginal granulocyte pool (MGP) has been measured in 70 normal male subjects. Perturbations were produced in the system with prednisone, physical exercise, epinephrine and bacterial endotoxin, and the influence of these perturbations on the above parameters was studied.

2. The mean values ± 1 SD, expressed as number of cells $\times 10^7$ per kilogram of body weight were as follows: TBGP, 65.0 ± 22.4; CGP, 31.7 ± 11.1; MGP, 33.3 ± 16.0. The mean value for the half-time disappearance rate ($T_{\frac{1}{2}}$) was 6.6 ± 1.16 hours, and for the GTR, 179.9 ± 74.3 $\times 10^7$ granulocytes per kg of body weight per day.

3. Prednisone increased the size of the CGP, the MGP and the $T_{\frac{1}{2}}$. The GTR was not altered.

4. Physical exercise and epinephrine produced a shift of cells from the MGP to the CGP. The size of the TBGP, the $T_{\frac{1}{2}}$ and the GTR was not influenced.

5. Bacterial endotoxin produced a shift of cells from the CGP to the MGP and an influx of cells into the TBGP from the intramedullary pool of granulocytes.

ACKNOWLEDGMENTS

The cooperation of Dr. William Knott and Warden John Turner of the Utah State Prison is very much appreciated. We are also grateful to those inmates of the Utah State Prison who volunteered for these studies. Mrs. Roseann Moore, Mrs. Joyce Rose and Miss Doris Kurth provided valuable technical assistance. We are grateful to Dr. M. H. Kuizenga, Upjohn Co., Kalamazoo, Mich., for the supplies of heparin sodium; to Dr. D. M. Monroe, Dow Corning, Corp., Los Angeles, Calif., for the silicone 4141; to Dr. G. M. Karus, Film Dept., E. I. DuPont de Nemours and Co., Wilmington, Del., for the Mylar; to Dr. J. H. Brewer, Hynson, Westcott and Dunning, Baltimore, Md., for the supply of crude cobra venom; and to Dr. Fred H. Schultz, Jr., The Wander Co., Chicago, Ill., for the bacterial endotoxin (Lipexal, SA 1064).

REFERENCES

1. Mauer, A. M., Athens, J. W., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. Leukokinetic studies. II. A method for labeling granulocytes *in vitro* with radioactive diisopropylfluorophosphate (DFP^{32}). J. clin. Invest. 1960, **39**, 1481.
2. Athens, J. W., Raab, S. O., Haab, O. P., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. Leukokinetic studies. III. The distribution of granulocytes in the blood of normal subjects. J. clin. Invest. 1961, **40**, 159.
3. Athens, J. W., Mauer, A. M., Raab, S. O., Haab, O. P., and Cartwright, G. E. Studies of granulocyte kinetics (abstract). J. clin. Invest. 1960, **39**, 969.
4. Raab, S. O., Haab, O. P., Athens, J. W., Ashenbrucker, H., and Cartwright, G. E. Endotoxin induced alterations in granulocyte kinetics. Clin. Res. 1961, **9**, 95.
5. Athens, J. W., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. Leukokinetic studies. I. A method for labeling leukocytes with diisopropylfluorophosphate (DFP^{32}). Blood 1959, **14**, 303.
6. Comar, C. L. Radioisotopes in Biology and Agriculture, Principles and Practice. New York, McGraw-Hill, 1955, p. 18.

7. Marsh, D. J., and Neale, E. A colorimetric method for the detection and determination of certain acid halide and acid anhydride compounds. Chem. and Ind. 1956, **22**, 494.
8. Craddock, C. G., Jr., Perry, S., Ventzke, L. E., and Lawrence, J. S. Evaluation of marrow granulocytic reserves in normal and disease states. Blood 1960, **15**, 840.
9. Killmann, S. A., Cronkite, E. P., Fliedner, T. M., and Bond, V. P. Erythro- and granulocytopoietic turnover in human bone marrow estimated from mitotic indices. Blood. In press.
10. Osgood, E. E. Control of peripheral concentration of leukocytes *in* Homeostatic Mechanisms. Brookhaven Symposia in Biology, no. 10. Washington, D. C., Dept. of Commerce, 1957, p. 31.
11. Patt, H. M., and Maloney, M. A. Control of granulocyte formation. *Ibid.*, p. 75.
12. Patt, H. M., Maloney, M. A., and Jackson, E. M. Recovery of blood neutrophils after acute peripheral depletion. Amer. J. Physiol. 1957, **188**, 585.
13. Mauer, A. M., Athens, J. W., Warner, H. R., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. An analysis of leukocyte radioactivity curves obtained with radioactive diisopropylfluorophosphate (DFP^{32}) *in* The Kinetics of Cellular Proliferation, F. Stohlman, Jr., Ed. New York and London, Grune and Stratton, 1959, p. 231.

STUDIES OF THE RATE OF DISAPPEARANCE OF LABELED THYROXINE FROM THE INTRAVASCULAR COMPARTMENT*

By EDWARD J. LENNON, NORMAN H. ENGBRING AND WILLIAM W. ENGSTROM

(From the Department of Medicine, Marquette University School of Medicine and Milwaukee County Hospital, Milwaukee, Wis.)

(Submitted for publication June 23, 1960; accepted March 2, 1961)

The reversible combination of thyroxine with certain of the plasma proteins clearly provides an efficient transport mechanism enabling microgram quantities of the hormone to be carried to tissue sites with minimal losses via glomerular filtration and other nonselective excretory pathways. The demonstration of thyroxine-binding proteins in other extracellular fluids (1) and within cells (2) suggests a means by which thyroxine may be selectively distributed to tissues according to their individual needs.

The role, if any, of the plasma thyroxine-binding proteins in various thyroid disorders is not clear. *In vitro* studies of the protein-thyroxine complex involve alterations of pH, P_{CO_2}, temperature and other aspects of the biochemical environment and may not give an accurate index of *in vivo* relationships. Furthermore, such studies neglect interactions of plasma thyroxine-binding proteins and extravascular thyroxine-binding proteins.

It seemed reasonable to suppose that the rate of disappearance of I^{131}-labeled L-thyroxine (T_4*) from the intravascular compartment during an *acute* experiment might yield information about the rate at which equilibrium between intra- and extravascular compartments was approached. The rate of disappearance should reflect the ability of the serum proteins to bind thyroxine and retain it within the vascular compartment in the face of all ambient forces favoring its removal. Conventional studies of labeled thyroxine survival (measured in days) are presumed to measure primarily the rate of thyroxine disposal *after* equilibration between labeled and stable thyroxine has been attained throughout the body. In preliminary studies, attempts were made to measure the rate of disappearance of T_4* from the circulation of isolated extremities but technical problems precluded accurate measurement. A whole-body technic was therefore employed and found to be satisfactory.

The method consisted in determining the rate of disappearance of I^{131}-L-thyroxine from the intravascular compartment of human subjects during a 30-minute period after allowing 20 minutes from time of intravenous injection for thorough intravascular mixing. The unsteady state prevailing shortly after the injection of T_4* was deliberately selected for the period of study because the rate of loss of T_4* from the circulation is maximal at this time and the apparent volume of distribution is rapidly expanding. The rate of disappearance was calculated and was shown to be reproducible.

Experimental results indicate: *a*) a profound influence of the liver on the acute disappearance rate of T_4*, indicated by a markedly prolonged intravascular retention of thyroxine in subjects with hepatic disease; *b*) rapid disappearance of T_4* in patients with diffuse toxic goiter (Graves' disease), but no significant change from normal in subjects fed large doses of desiccated thyroid, and slight prolongation of disappearance rate in subjects with toxic nodular goiter; and *c*) marked prolongation of disappearance rate in subjects with spontaneous myxedema, but *no* similar prolongation in subjects with Graves' disease who developed myxedema after treatment of hyperthyroidism.

MATERIAL AND METHODS

Each subject was evaluated by one or more of the authors and a clinical impression of the state of thyroidal function was made without reference to laboratory studies. Impressions were confirmed by serum protein-bound iodine (PBI) determinations and, in some instances, by measuring the thyroidal 24-hour uptake of I^{131}. The

* This investigation was supported in part by Public Health Service Grants A-1200 and 2A-5023, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

control group consisted of 15 euthyroid subjects ranging from 13 to 76 years of age. None had clinical evidence of hepatic or renal disease, but some suffered from a variety of other nonfebrile illnesses, e.g., generalized arteriosclerosis, carcinoma of the lung, mild malnutrition, compensated heart disease, and so forth.

The study groups consisted of 9 euthyroid subjects with liver disease, 7 subjects with toxic nodular goiter, 9 with Graves' disease and thyrotoxicosis, 4 subjects with Graves' disease who had become euthyroid after treatment (one of these receiving 3 grains of desiccated thyroid daily because of moderate exophthalmos), 5 subjects with Graves' disease who were myxedematous as a result of treatment and 7 subjects with spontaneous primary myxedema. In addition, 10 subjects whose ranges of acute thyroxine disappearance rate approximated the ranges observed among the various groups were subjected to a second study 2 to 4 days after the initial measurement to estimate reproducibility of the method.

Subjects were brought to the radioisotope laboratory in the early afternoon without special preparation. A no. 18 needle was inserted and secured in an antecubital vein and connected to one limb of a Radicoil (Abbott) resting in a shielded well-type scintillation counter. The other limb of the Radicoil was connected via a three-way stopcock to a 20 ml syringe and a solution of 50 mg aqueous heparin in 500 ml of isotonic saline. Blood was withdrawn through the Radicoil to the 10 ml mark of the syringe, counted for 1 minute, and reinjected into the subject. This step was repeated one or more times, depending on background radioactivity. At time zero, a 20 to 30 μc dose of T_4* of high specific activity (Abbott) was injected into a vein in the opposite arm. The T_4* was used within 7 days of delivery; it was not rechromatographed before use since prior studies indicated that free I^{131} had little influence on the function being measured. The three-way stopcock was adjusted to permit a slow drip of the heparin solution to prevent clotting in the indwelling needle. At 5-minute intervals, blood was drawn into the Radicoil, counted for 1 minute and reinjected. Care was taken to draw the blood slowly (20 seconds) to the 10 ml mark of the syringe to prevent variations in mixing and dilution. Counting at 5-minute intervals was continued for 50 to 60 minutes after injection of the T_4*. There was no significant adherence of thyroxine to the plastic Radicoil in the presence of plasma proteins.

RESULTS

When the curve of thyroxine disappearance is derived on semilog paper by plotting the net counts per minute (log scale) against time (linear scale), three distinct components of the curve are apparent. The first component, with most rapidly declining concentration of T_4*, extends from 0 to 10 or 20 minutes after injection and primarily represents mixing and dilution of the T_4* within the vascular compartment. In normal subjects this brief, initial phase requires less than 10 minutes; however, when circulation is slowed, as in myexedma, it may extend to 20 minutes. Even in this brief period of mixing, simultaneous egress of T_4* from the circulation will occur, but it is not possible to separate that decline in radioactivity due to mixing from that due to extravascular movement. The moment when mixing is complete and when the decrements of T_4* largely represent egress from the circulation, cannot be ascertained.

The second component is curvilinear and extends for 1 or 2 days. It primarily represents mixing of T_4* throughout the body with establishment of an equilibrium between labeled and stable thyroxine. The curvilinear regression clearly represents the *simultaneous* operation of a number of processes: *a*) egress from the circulation—the rate of fall in plasma radioactivity by this function will be greatest when intravascular concentration of T_4* is highest, i.e., the early part of the curve; *b*) re-entry of T_4* into the circulation from the extravascular compartment—as extravascular concentrations of T_4* slowly increase, the contribution to plasma radioactivity increases; *c*) superimposed is the third component of the curve—the disposal of thyroxine from the body.

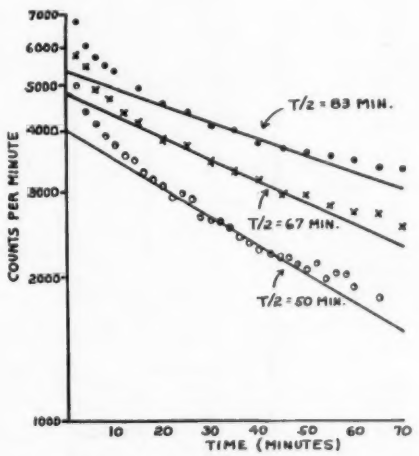


FIG. 1. THREE ILLUSTRATIVE CURVES PLOTTED ON SEMILOG PAPER SHOWING THE REGRESSION OF NET COUNTS PER MINUTE ON TIME AFTER INTRAVENOUS INJECTION OF I^{131}-L-THYROXINE. THE HALF-TIME VALUE IS DERIVED FROM CALCULATION OF THE SLOPE OF THAT SEGMENT OF THE CURVE BETWEEN 20 AND 50 MINUTES.

TABLE I

*Replicate determinations of acute thyroxine disappearance**

Subject	Acute thyroxine disappearance rate Half-time: First study	Second study	Difference	Difference
	min	*min*	*min*	%
A.K.	57.8	56.5	− 1.3	− 2.30
R.B.	43.9	44.1	+ 0.2	+ 0.46
B.P.	71.8	78.8	+ 7.0	+ 9.75
E.K.	96.5	88.3	− 8.2	− 8.50
F.H.	114.0	109.1	− 4.9	− 4.30
C.B.	65.4	68.1	+ 2.7	+ 4.13
H.S.	59.1	54.5	− 4.6	− 7.78
F.T.	123.4	103.8	−19.6	−15.88
L.J.	99.7	84.6	−15.1	−15.14
L.B.	55.6	63.5	+ 7.9	+14.21
Mean	78.7	75.1	− 3.6	− 2.5
SD†			± 8.9	± 9.9

* Second studies were performed 2 to 4 days after initial studies.

† Standard deviation.

The third component is practically linear and can be measured after equilibration is established (3). The normal half-time of thyroxine "survival" or disposal is about 6 days. Since the half-time is 3 or more days even in hyperthyroid states, the slope of this linear component, when considered over a 30-minute period, is almost imperceptible and could influence calculated results very little.

Thus, while all of the above simultaneously occurring processes can not be separated, to obtain some measure or approximation of the net effect of forces favoring retention and removal of intravascularly injected (initially unbound) T_4*, attention must be directed to the interval when T_4* concentrations are highest and decreasing most rapidly. While the mixing phase is rapid in normals, it may require as long as 20 minutes in some patients with myxedema. Therefore, so as to be able to compare different groups in a similar manner and since the "rate" was obtained by calculation rather than a visual fit, the regression from 20 to 50 minutes was chosen for analysis (Figure 1 and Table II). In this interval mixing should have been completed, re-entry of T_4* from extravascular spaces should be small although perhaps not insignificant in all circumstances, and

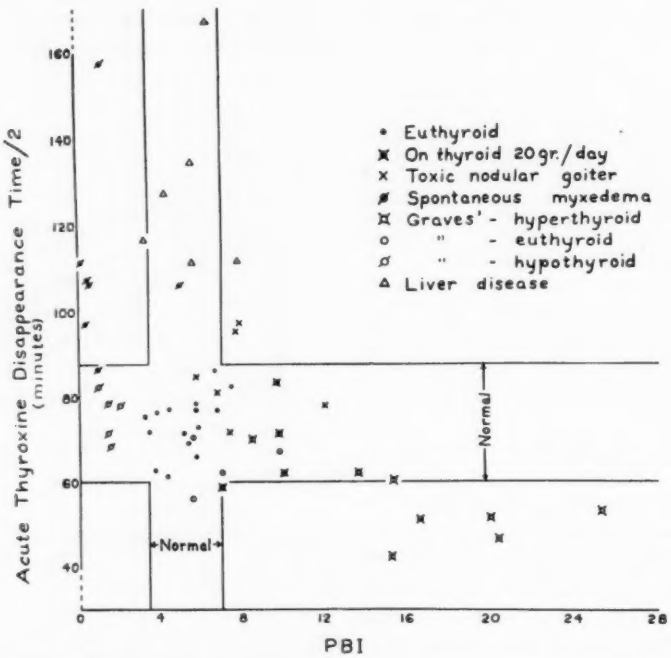


FIG. 2. THE RELATIONSHIP BETWEEN PBI, REFLECTING LEVELS OF THYROIDAL ACTIVITY, AND THE ACUTE THYROXINE DISAPPEARANCE RATE.

TABLE II

Experimental results for control subjects and study groups

Group	Subject	Age	Sex	Race	PBI	Thyroidal 24 hr. I^{131} uptake	Acute thyroxine disappearance rate Half-time
		yrs			*μg%*		*min*
Healthy, euthyroid	M.P.	34	F	N.	5.2		71.8
	E.R.	29	F	Cau.	3.8	21.9	63.1
	A.D.	44	M	Cau.	3.9		76.6
	A.Da.	75	M	Cau.	5.8		77.4
	D.D.	28	M	Cau.	3.3		75.8
	D.E.	13	M	Cau.	7.5		82.7
	D.Z.	48	F	Cau.	5.8		78.6
	I.J.	42	F	N.	4.5	10.6	77.4
	L.C.	47	M	Cau.	3.5		72.0
	M.S.	76	M	Cau.	5.9		73.2
	P.W.	53	M	Cau.	4.4		61.6
	W.S.	36	M	N.	5.8	18.5	66.2
	R.Q.	25	M	Cau.	5.4	10.7	69.4
	C.A.	29	M	N.	6.7	21.6	86.5
	G.K.	42	M	Cau.	6.8	19.5	77.2
						Mean	74.0
						SD ±	6.9
Toxic nodular goiter	A.K.	50	F	Cau.		56.2	84.1
	E.A.	77	F	Cau.	5.8	42.1	85.0
	M.H.	70	F	Cau.	7.4	54.4	72.0
	L.K.	68	F	Cau.	7.7	44.2	95.6
	I.A.	76	F	Cau.	12.0	48.5	78.4
	A.S.	65	F	Cau.	7.9	32.4	97.7
	E.K.	49	F	Cau.	6.8	49.0	82.2
						Mean	85.0
						SD ±	9.1
Graves' disease, toxic	M.R.	21	F	N.	16.6		51.3
	S.B.	67	M	Cau.	20.0		51.9
	M.G.	39	F	N.	8.5	36.6	70.3
	S.T.	54	F	Cau.	15.3	71.5	60.7
	M.S.	26	F	Cau.	15.2	67.4	42.6
	D.A.	32	F	Cau.	13.6	43.0	62.4
	E.H.	54	M	Cau.	25.3	61.5	53.3
	K.M.	47	F	Cau.	20.2	66.0	46.9
	M.P.	30	F	Cau.		40.5	56.5
						Mean	55.1
						SD ±	8.4
Graves' disease, euthyroid	R.B.	27	M	Cau.	7.0	38.2	62.3
	S.T.	54	F	Cau.	5.6	11.8	70.7
	V.H.	28	F	Cau.	9.9		67.5
	K.S.	48	F	Cau.	5.8		56.2
						Mean	64.2
						SD ±	6.3
Graves' disease, myxedema	S.N.	19	F	Cau.	1.5	8.4	78.8
	M.R.	34	F	Cau.	1.0	5.0	82.5
	B.W.	30	F	Cau.	2.1		78.2
	D.A.*	32	F	Cau.	1.5	0.4	71.8
	E.C.	56	F	Cau.	1.6	5.9	68.6
						Mean	76.0
						SD ±	5.6

* Previously studied while thyrotoxic.

TABLE II—(*Continued*)

Group	Subject	Age	Sex	Race	PBI	Thyroidal 24 hr I^{131} uptake	Acute thyroxine disappearance rate Half-time
		yrs			*μg%*		*min*
Spontaneous primary myxedema	E.M.	71	F	Cau.	1.3		157.6
	G.E.	75	F	Cau.	3.0	2.4	106.4
	H.B.	69	F	Cau.	0.6	1.2	106.4
	C.D.	68	F	Cau.	0.5	3.2	107.5
	O.S.	59	M	Cau.	1.0		86.5
	M.B.	55	F	N.	0.4	1.4	97.1
	S.C.	85	F	Cau.	0.2	4.3	111.5
						Mean	110.4
						SD	± 22.4
Liver disease	J.H.†	24	M	Cau.	7.8		111.9
	L.T.	43	M	Cau.	4.3		127.6
	R.L.	42	M	Cau.	6.4		250.9
	O.T.	42	M	Cau.			273.7
	A.W.	46	F	N.	5.6		135.0
	F.T.	48	M	Cau.	3.3		116.7
	M.W.	67	M	Cau.	5.6	15.4	111.5
	J.E.	44	M	Cau.			95.9
	M.B.	44	F	N.			248.8
						Mean	163.6
						SD	± 71.8
Normals fed thyroid	W.S.	36	M	N.	9.8		71.7
	R.Q.	25	M	Cau.	7.0		59.0
	C.A.	29	M	N.	10.0		62.4
	G.K.	42	M	Cau.	9.7		83.8
						Mean	69.2
						SD	± 11.1

† Studied during acute phase of infectious hepatitis.

the effects of thyroxine disposal from the body as a whole should be negligible.[1]

Statistical analysis indicated the probability that the portion of the curve for the relatively short 20 to 50 minute interval was linear in each case ($p < 0.01$). This linear portion of the regression curve was solved mathematically in each instance by the method of least squares and the slope was determined ($\log\ y = a + bx$). The time required for the net counts per minute to decline to one-half the intercept value at time zero (time of injection of T_4*) was obtained by extrapolation and is expressed as the "acute thyroxine disappearance half-time." The statistical methods used were those described by Snedecor (4).

Replicate studies. To determine reproducibility in the same subject, ten subjects were restudied after 2 to 4 days without treatment. Subjects were selected on the basis of the first study to include the range of values noted within the study groups. Table I compares the results of first and second studies. The mean of the difference (first study minus second study) was − 3.6 minutes. Group means for first and second studies were not statistically different ($p > 0.50$).

Euthyroid controls. The acute thyroxine disappearance rate for 15 euthyroid controls was 74.0 minutes. Analysis of covariance indicated the likelihood of a common population regression coefficient for these subjects. Variations correlated with age, sex or race could not be detected in this relatively small group. Individual data for subjects in this and subsequent groups are given in Table II and in Figure 2.

[1] Our data, which does not contain simultaneously determined concentrations of extravascular T_4*, does not permit the calculation of a precise rate constant for transfer of T_4 out of plasma, either in normals or in the different disorders studied. This would also require knowledge of the rate constant for transfer of T_4 from the extravascular compartment to plasma. Thus, our results are expressed merely as a "rate," rather than a rate constant, and the slopes can not be considered to be true zero-time slopes.

Liver disease. Marked prolongation of thyroxine disappearance rate was noted in the eight patients with Laennec's cirrhosis and in the one with acute infectious hepatitis. Details as to degree of hepatic involvement are given in Table III. The mean half-time of disappearance was 163.6 minutes. The slowest disappearance rates were observed in the subjects with ascites, indicating that expansion of the extracellular fluid volume need not accelerate disappearance rate (as might be expected if this measured simple diffusion from the intravascular compartment). The wide range of values observed undoubtedly reflects different degrees of hepatic impairment, but could not be correlated with any of the recorded indices of liver function. Difference from the control group was highly significant ($p < 0.001$).

Hyperthyroidism. A sharp distinction between patients with nodular toxic goiter and those with diffuse toxic goiter (Graves' disease) was evident. The mean half-time of thyroxine disappearance for seven subjects with toxic nodular goiter (85.0 minutes) was actually significantly longer than that observed in the controls ($p < 0.01$). By contrast, nine patients with diffuse toxic goiter had a mean thyroxine disappearance half-time of 55.1 minutes, very significantly *shorter* than the control group ($p < 0.001$).

In an effort to clarify this difference four healthy, euthyroid subjects were given increasing amounts of desiccated thyroid over a 6-week period, up to 20 grains daily. The thyroxine disappearance rate was determined at 2-week intervals. The results are summarized in Table II. As noted by others (5–7), the subjects tolerated large doses of thyroid well, although they manifested weight loss, slight tremor, increased sweating and mild tachycardia. The highest PBI value produced was 10.3 μg per 100 ml. Mean thyroxine disappearance rate at the end of the experimental period (after 20 grains of desiccated thyroid had been administered daily for 1 week) was 69.2 minutes. This was not significantly different from the euthyroid control group ($p > 0.40$) or from the results in the same subjects before treatment with desiccated thyroid. None of the subjects had changes in disappearance rate falling outside the 95 per cent confidence limits for replicate studies in untreated subjects.

Myxedema and treated hyperthyroidism. Mean half-time of acute thyroxine disappearance for seven subjects with spontaneous, primary myxedema was 110.4 minutes, a highly significant difference from the control group ($p < 0.001$). When five subjects with Graves' disease were studied after they had become profoundly myxedematous from treatment, their thyroxine disappearance rate (mean half-time of 76.0 minutes) was very significantly longer ($p < 0.001$) than in hyperthyroid subjects with Graves' disease (mean half-time of 55.1 minutes), but not significantly different from that of euthyroid controls. Yet, interestingly, the disappearance half-time of 76.0 minutes was significantly shorter than in those with spontaneous myxedema (mean 110.4 minutes). Thus, although the development of myx-

TABLE III

Indices of hepatic function in subjects with liver disease

Subject	Age	Sex	Race	Ascites	BSP retention at 45 min	Serum albumin	Prothrombin time	Hgb.	Cholesterol	PBI	Acute thyroxine disappearance rate Half-time
	yrs				*%*	*g%*	*sec*	*g*	*mg%*	*μg%*	*min*
L.T.	43	M	Cau.	+	40.6	1.91	12.7	9.5	140	4.3	127.6
A.W.	46	F	N.	0	34.8	1.84	16.4	8.5	348	5.6	135.0
J.E.	44	M	Cau.	0	31.4	3.15	19.0	10.0	100		95.9
J.H.*	24	M	Cau.	0	†	3.90	13.4	14.0	215	7.8	111.9
O.T.	42	M	Cau.	+	42.8	3.60	13.4	7.0	308		273.7
M.W.	67	M	Cau.	0	41.0	2.42	15.6	9.0	130	2.6	111.5
F.T.	48	M	Cau.	0			12.4	15.0		3.3	116.7
R.L.	42	M	Cau.	+	†	2.30	24.6	10.5	133	6.4	250.9
M.B.	44	F	Cau.	+	†	2.20	28.5	9.0	138		248.8

* Acute infectious hepatitis.
† Icteric.

edema slows the disappearance rate, a specific difference in handling of labeled thyroxine seems to exist in Graves' disease even when functional thyroid tissue is absent, or virtually so.

Euthyroid Graves' disease. Four subjects with Graves' disease were studied when they were euthyroid after treatment. One of these (V.H.) was maintained on 3 grains of desiccated thyroid daily because of moderately severe exophthalmos (the elevated PBI noted in this case was attributed to extraneous iodine ingestion). Subjects had been euthyroid for from 3 months to 2 years. The group mean was 64.2 minutes and differed from the control group at marginal significance level ($p < 0.05$).

DISCUSSION

The early phases of disappearance of intravenously injected T_4* from the circulation have not been studied in detail. Limited data from other sources (3, 8) and observations during the course of the present studies implicate a complex series of physiological events, many of which proceed simultaneously. These events include: *a*) mixing of T_4* within the intravascular compartment with almost immediate binding to the plasma proteins; *b*) distribution throughout the extrathyroidal thyroxine pool with intravascular-extravascular competition for T_4*; *c*) specific trapping of thyroxine by certain organs, particularly the liver; *d*) disposal of T_4* from the body via the gastrointestinal tract, by excretion of small amounts in the urine (8) and by metabolic degradation with subsequent appearance of free I^{131} in the blood, the thyroid and the urine. In the present studies, covering only a 30-minute period, it is unlikely that degradation of T_4* is significant since the total turnover of endogenous thyroxine does not appear to exceed 30 per cent per day even in hyperthyroid states (9). The present studies are believed to reflect primarily the events of *b* and *c* above, namely, the early distribution and specific trapping of thyroxine.

The half-time value determined from decreasing blood levels of radioactivity in each case reflects the rate at which equilibrium between labeled and stable thyroxine is being approached within the body. This process of equilibration is occurring at different rates between the intravascular and the many extravascular compartments of the body. The method of measurement employed in the present studies fails to distinguish between these various rates, but reflects them collectively. The technique intends only to estimate the balance existing between forces regulating the rate of removal of T_4* from the circulation. Although the calculated value does not represent a true rate constant, the results obtained were reproducible among groups of euthyroid individuals and among individuals with the various pathological states noted, and the results were statistically different between certain groups.

Factors which influence the acute rate of disappearance of a tracer amount of T_4* from the blood include: *a*) the relative size of intra- and extravascular thyroxine pools (e.g., a large intravascular pool of thyroxine would slow the rate of disappearance); *b*) the relative thyroxine-binding affinity and saturation of intra- and extravascular proteins (e.g., increased intravascular thyroxine-binding capacity would be associated with a slower rate of removal of T_4*); *c*) the integrity of the vascular lining which prevents extravasation of protein-bound T_4*; and *d*) the ability of certain organs specifically to concentrate protein-bound or free T_4*.

The relationship of intravascular protein binding of thyroxine to thyroidal economy in normal as well as in pathological states has received considerable attention. *In vitro* measurements of thyroxine-binding capacity permit comparison of the binding affinity of plasma proteins in normal and abnormal states under the conditions imposed by the technique of measurement. The effectiveness with which plasma proteins retain thyroxine within the circulation of intact subjects, however, must be equally dependent upon the relative binding affinity of extravascular proteins as well as upon the other factors mentioned above. The present studies suggest a reasonably good correlation between the rate of removal of thyroxine from the blood and *in vitro* estimates of plasma-binding capacity. In circumstances reported to exhibit increased thyroxine-binding capacity of the serum proteins, a delay in the acute disappearance of thyroxine from the circulation was observed, i.e., in seven patients with primary myxedema, in a single subject with acute hepatitis, and in subjects given diethylstilbestrol (unpublished observations).

The findings in subjects with various types of hyperthyroidism are of particular interest. Subjects with Graves' disease, at all levels of thyroid function, had more rapid thyroxine disappearance rates than other subjects with comparable levels of metabolic activity. While it is clear, in comparing hyperthyroid, euthyroid, and hypothyroid subjects with Graves' disease, that hyperthyroidism has some influence on the function measured, the failure to find significant similar acceleration in subjects with toxic nodular goiter or in thyrotoxicosis medicamentosa suggests a more specific abnormality in Graves' disease. It may be argued that subjects with Graves' disease simply have a larger extravascular pool of thyroxine or that there was greater saturation of binding proteins (since PBI levels were somewhat higher in the Graves' group). However, the persistence of accelerated disappearance rate, in relation to others with comparable metabolic status, in Graves' subjects made hypothyroid, and probably also in those made euthyroid, invalidates this argument. Also, it is likely that those individuals with toxic nodular goiter and thyrotoxicosis medicamentosa had ample time to expand their extravascular thyroxine pools to a degree comparable to hyperthyroid Graves' subjects.

The data support the concept of a qualitative, biological difference between Graves' disease and toxic nodular goiter and suggest altered *in vivo* plasma or possibly even tissue protein binding of thyroxine in Graves' disease. Indeed, the abnormality is apparent when these patients have become myxedematous after treatment. In a preliminary report, Richards, Dowling and Ingbar (10) noted that thyroxine-binding by prealbumin is usually decreased in untreated thyrotoxic patients with Graves' disease, but a similar defect in subjects ill with other diseases was also observed. It may be that defective prealbumin binding of plasma in Graves' disease plays a significant role in the phenomena observed in the acute thyroxine disappearance rate. Our data and those of others do not exclude the possible importance of extravascular forces. Since we have not observed the acute thyroxine disappearance rate to be accelerated in illnesses other than Graves' disease, the defective prealbumin binding associated with some nonspecific illnesses may in turn be balanced by a comparable decrease in extravascular thyroxine-binding capacity. Certainly, patients with nonspecific illnesses do not exhibit signs of hyperthyroidism.

Our observations on acute thyroxine disappearance rate in Graves' disease are consonant with the increased thyroxine turnover rates in subjects with treated Graves' disease (11) and with the finding that heterologous serum labeled with T_4* disappears from the circulation in accordance with the recipient's thyroid status rather than the donor's (12). Also, Hamolsky, Ellison and Freedberg have shown that labeled thyroxine is more avidly incorporated by guinea pig diaphragm *in vitro* from sera of patients with Graves' disease than from sera of subjects with toxic nodular goiter or sera from normals enriched *in vitro* to hyperthyroid levels of PBI (13). By contrast, they found that labeled thyroxine mixed with sera from subjects with Graves' disease disappeared more rapidly from the circulation of recipient dogs than when mixed with sera from normals or subjects with toxic nodular goiter. This finding is not easily interpreted without knowledge of the relative binding affinity for thyroxine of dog and human serum or of dog's extravascular proteins. Their findings may reflect poorer thyroxine binding by Graves' plasma and represent distributional phenomena rather than peripheral metabolic factors.

The finding of an actual, although slight, prolongation of thyroxine disappearance rate in subjects with toxic nodular goiter may be due to expansion of intravascular pool size out of proportion to increase in turnover. It is equally possible that this prolongation is related to the increased age (although other older patients did not share this finding) or to minor impairment of hepatic function (clinically not apparent).

The acute thyroxine disappearance rate was consistently and markedly prolonged in subjects with cirrhosis of the liver; in most instances the disappearance rates were even longer than in patients with spontaneous myxedema. While increased binding of thyroxine by plasma globulins has been described in hepatitis where the PBI is often elevated (14), this seems unlikely in Laennec's cirrhosis. In the latter, the PBI is usually normal or even low, and increased red cell uptake of triiodothyronine *in vitro* has been reported (15). The latter finding has been attributed to

decreased binding affinity by the serum proteins in this condition. If such is the case in most instances of cirrhosis, it would then seem that intravascular factors (e.g., the ability of the plasma proteins to bind thyroxine) are of much less importance than are extravascular phenomena in effecting the acute thyroxine disappearance rate in liver disease. Thus, the liver may play a more important role in the handling and metabolism of thyroxine than has generally been appreciated. Preliminary observations during these studies and observations by others (8) indicate that thyroxine rapidly accumulates in the liver. Since fecal excretion of organic iodine is small (9, 16), this must indicate that: *a*) thyroxine trapped by the liver is excreted in the bile and reabsorbed via the enterohepatic circulation; *b*) thyroxine is released to the circulation after hepatic trapping, free or in bound form; or *c*) the liver stores large amounts of thyroxine for its own metabolic needs. Quantitative data are not available to decide which of the possibilities might predominate in affecting the acute handling of thyroxine.

The results of the present studies are very similar to those recently reported by Peterson and Pierce (17) regarding the disappearance of cortisol, cortisone and corticosterone from the intravascular space. Alterations from normal in the rates of disappearance of these steroids were noted in cirrhosis, myxedema, thyrotoxicosis [2] and after administration of estrogen to normal subjects. These qualitative changes were similar to those we have reported for I^{131}-thyroxine.

SUMMARY AND CONCLUSIONS

1. A method is described for the determination of the rate at which equilibrium between intra- and extravascular thyroxine pools is approached after intravenous administration of labeled thyroxine. This measurement, termed the "acute thyroxine disappearance rate" is accomplished during a 30-minute period beginning 20 minutes after the intravenous injection of I^{131}-labeled L-thyroxine.

2. Subjects with Graves' disease were found to have shorter acute thyroxine disappearance rates at all levels of thyroid function than other subjects with comparable thyroidal activity. Even when functionally athyrotic, the patients with treated Graves' disease handled thyroxine differently from athyrotic subjects without Graves' disease. This observation suggests that plasma or extravascular protein binding of thyroxine in subjects with Graves' disease may be altered *in vivo* and that this alteration is not the direct result of hypermetabolism or hyperthyroidism.

3. No significant shortening of thyroxine disappearance rate was noted in patients with toxic nodular goiter or in normals fed exogenous thyroid. This finding indicates a qualitative difference between the toxic nodular goiter and Graves' disease. These observations cannot be accounted for by differences in the level of the PBI or derivatives of this measurement, since the thyroxine disappearance rate in Graves' disease remained relatively rapid after treatment of hyperthyroidism.

4. Greatly prolonged thyroxine disappearance rates in subjects with liver disease suggest that the liver plays an important role in the handling of thyroxine.

5. Replicate experiments indicate that the method of study is reproducible and applicable to the study of drug and hormone effects on the rate of egress of labeled hormone from the circulation.

[2] By personal communication, we are informed that all of their patients with "thyrotoxicosis" suffered from Graves' disease.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Miss Virginia Griffin and Mrs. Marian Wisniewski and to thank Miss Griffin for reviewing the statistical analyses.

REFERENCES

1. Freinkel, N., Ingbar, S. H., and Dowling, J. T. The influence of extracellular thyroxine-binding protein upon the accumulation of thyroxine by tissue slices. J. clin. Invest. 1957, **36**, 25.
2. Tata, J. R. A cellular thyroxine-binding protein fraction. Biochim. biophys. Acta 1958, **28**, 91.
3. Haddad, H. M. Rates of I^{131}-labeled thyroxine metabolism in euthyroid children. J. clin. Invest. 1960, **39**, 1590.
4. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 5th ed. Ames, Iowa State College Press, 1956.
5. Johnston, M. W., Squires, A. H., and Farquharson, R. F. The effect of prolonged administration of thyroid. Ann. intern. Med. 1951, **35**, 1008.

6. Goldsmith, R., and Stanbury, J. B. The tolerance of patients with myxedema for thyroid. J. clin. Endocr. 1955, **15**, 568.
7. Danowski, T. S., Huff, S. J., Tarail, R., Wirth, P., Peters, J. H., Mateer, F. M., and Garver, K. Serum protein-bound iodine during ingestion of desiccated thyroid. J. clin. Endocr. 1952, **12**, 1572.
8. Benua, R. S., Albert, A., and Keating, F. R., Jr. The metabolism of radiothyroxine in exophthalmic goiter. J. clin. Endocr. 1952, **12**, 1461.
9. Ingbar, S. H., and Freinkel, N. Simultaneous estimation of rates of thyroxine degradation and thyroid hormone synthesis. J. clin. Invest. 1955, **34**, 808.
10. Richards, J. B., Dowling, J. T., and Ingbar, S. H. Alterations in the plasma transport of thyroxine in sick patients and their relation to the abnormality in Graves' disease (abstract). J. clin. Invest. 1959, **38**, 1035.
11. Ingbar, S. H., and Freinkel, N. Studies of thyroid function and the peripheral metabolism of I^{131}-labeled thyroxine in patients with treated Graves' disease. J. clin. Invest. 1958, **37**, 1603.
12. Levy, R. P., Kelly, L. W., Jr., Cooper, G. W., and Jefferies, W. McK. The volume of distribution and turnover of endogenously labelled human thyroid hormone from euthyroid and hyperthyroid donors (abstract). J. clin. Invest. 1955, **34**, 948.
13. Hamolsky, M. W., Ellison, H. E., and Freedberg, A. S. The thyroid hormone-plasma protein complex in man. I. Differences in different states of thyroid function. J. clin. Invest. 1957, **36**, 1486.
14. Vannotti, A., and Béraud, T. Functional relationships between the liver, the thyroxine-binding protein of serum, and the thyroid. J. clin. Endocr. 1959, **19**, 466.
15. Hamolsky, M. W., Golodetz, A., and Freedberg, A. S. The plasma protein-thyroid hormone complex in man. III. Further studies on the use of the *in vitro* red blood cell uptake of I^{131}-*l*-triiodothyronine as a diagnostic test of thyroid function. J. clin. Endocr. 1959, **19**, 103.
16. Berson, S. A., and Yalow, R. S. Quantitative aspects of iodine metabolism. The exchangeable organic iodine pool, and the rates of thyroidal secretion, peripheral degradation and fecal excretion of endogenously synthesized organically bound iodine. J. clin. Invest. 1954, **33**, 1533.
17. Peterson, R. E., and Pierce, C. E. The metabolism of corticosterone in man. J. clin. Invest. 1960, **39**, 741.

THROMBOPLASTIN GENERATION ACCELERATOR, A NEWLY RECOGNIZED COMPONENT OF THE BLOOD COAGULATION MECHANISM PRESENT IN EXCESS IN CERTAIN THROMBOTIC STATES*

By CHRIS A. PASCUZZI, JOHN A. SPITTEL, JR., JOHN H. THOMPSON, JR. AND CHARLES A. OWEN, JR. WITH THE TECHNICAL ASSISTANCE OF CHARLENE MATTHEES

(From the Sections of Medicine and Clinical Pathology, Mayo Clinic and Mayo Foundation, Rochester, Minn.)

(Submitted for publication October 31, 1960; accepted March 2, 1961)

Recent years have witnessed an increasing interest in the study of the possible relationship of blood coagulation to intravascular thrombosis (1–4). In spite of numerous methods of investigation, a consistent means of demonstrating and defining a clotting abnormality in thromboembolic diseases has not emerged.

By retarding artificially the thromboplastin generation test (TGT) we have been able to show acceleration of thromboplastin generation in certain patients with intravascular thrombosis (5, 6). Our experience to date indicates that 75 per cent of 52 patients who had arteriosclerosis obliterans with thrombosis demonstrated the acceleration, 50 per cent of 40 patients with "idiopathic" recurrent thrombophlebitis did likewise, but only 11 per cent of 35 patients who had arteriosclerosis obliterans without thrombosis exhibited acceleration of the TGT. Of several hundred "normal" plasmas, less than 1 per cent was abnormally rapid in the TGT. It became apparent that the cause of the rapid formation of thromboplastin in the blood of these patients was demonstrable only in the adsorbed plasma reagent of the TGT, not in the serum reagent. Since the adsorption of plasma removes plasma thromboplastin component (PTC or factor IX), stable factor (factor VII), and Stuart factor (factor X), as well as prothrombin, these substances are probably not directly involved in the clot acceleration. A number of other factors remains; these are considered in detail in the present report.

We wish to present evidence that the abnormal clot acceleration results from the presence of an excessive amount of coagulative activity which resides with the globulins in normal human plasma. We have designated this protein "thromboplastin generation accelerator," or simply "TGA," because its activity seems to lie in the thromboplastin generation phase of clotting.

METHODS

1. One-stage prothrombin time of Quick (7). This test, as modified locally (8), gives a normal value of 17 to 19 seconds. For the determinations of "dilute prothrombin time," fresh plasma was diluted 1:5 and 1:10 with oxalated saline solution (1 part of 0.1 M sodium oxalate and 9 parts of 0.9 per cent sodium chloride), and the test was carried out in the usual manner. Normal values are 30 to 49 seconds for the 1:5 dilution of plasma, and 56 to 67 seconds for the 1:10 dilution.

2. Plasma clotting (recalcification) time (9). The normal value is 90 to 110 seconds.

3. Thromboplastin generation test (TGT) of Biggs and Douglas (10), modified by Duckert and co-workers (11), with substitution of soybean phosphatides for platelets (12). Normal values are the shortest clotting times, 8 to 11 seconds, occurring at 7 to 11 minutes of incubation. Retardation of the TGT (6) is carried out by diluting the plasmatic reagent 1:50 with buffered saline [1 part of imidazole buffer (13) and 9 parts of 0.9 per cent sodium chloride] instead of our usual 1:25 dilution [Duckert modification (11)]. Normal values for the "retarded TGT" are the shortest clotting times, 13 to 16 seconds, occurring at 11 to 13 minutes of incubation. Since we have demonstrated excess TGA activity as well with soybean phosphatide as with platelets (5), the convenience of the former has led to its routine use.

4. Electrophoresis of plasmatic factors (14). The fresh plasma was first treated with barium sulfate (50 mg per ml, for 5 minutes at 24° C) to remove prothrombin and factors VII, IX and X; 0.15 ml was applied to strips of Whatman no. 2 filter paper, 4 cm wide. Electrophoresis was carried out in a cold room (4° C) for a period of 16 hours at a constant current of 0.6 ma per cm width of paper strips; the buffer used was sodium barbital-bar-

* This investigation was supported in part by research grant H-5008(R1) from the National Institutes of Health.

bituric acid of pH 8.6 and 0.05 ionic strength. The various cut-paper fractions were eluted with 2.5 ml of buffered saline.

5. Precipitating with ammonium sulfate. At room temperature, 1 ml of fresh barium sulfate-adsorbed plasma, diluted to a total volume of 10 ml, was subjected to progressively greater concentrations of saturated solution of ammonium sulfate after removal by centrifugation of the precipitate formed in each preceding step. The various precipitates were dissolved and subjected to dialysis against several changes of 0.9 per cent sodium chloride for 16 hours at 4° C.

MATERIALS

1. Excess TGA plasma. This was obtained from patients with arteriosclerosis obliterans and associated thrombosis. All of these samples of plasma exhibited increased thromboplastic activity in the thromboplastin generation tests whether the plasma was diluted 1:25 or 1:50. This increase in activity ranged from approximately two to five times the normal rate of generation of thromboplastin. These values were estimated by comparison with the thromboplastin-generating activity of normal plasma. Essentially, the thromboplastin generated at various intermediate intervals of time is calculated from serial dilutions of the control and patient plasmas. If, for example, at the fifth minute of incubation twice as much thromboplastin has been generated by the patient as by the control, the activity is recorded as two times normal.

On the other hand, in the studies of the properties of TGA (electrophoretic mobility, precipitation, and heat stability) an attempt at more direct evaluation of TGA activity was made by the degree of augmentation of the generation of thromboplastin by normal plasma to which the fraction was added.

2. AHG-deficient plasma. Fresh plasma was obtained repeatedly from 2 severely hemophilic patients. These samples were cross checked with samples from 6 other hemophilic patients and all 8 exhibited the same defect, a lack of the antihemophilic globulin (AHG or factor VIII).

3. Aged normal plasma. Fresh normal plasma was heated to 37.5° C for 24 to 30 hours until the prothrombin time exceeded 60 seconds and the plasma clotting time exceeded 300 seconds. This plasma has been shown to be deficient in both factor V and AHG when tested against plasmas of parahemophilic and hemophilic patients.

All samples of plasma were oxalated by the addition of 0.5 ml of 0.1 M sodium oxalate to 4.5 ml of freshly drawn whole blood.

RESULTS

Certain known coagulation factors are present in normal aged serum (factors VII, IX, X, Hageman and PTA); others are active in fresh plasma that has been treated with one of the various chemical adsorbents, such as barium sulfate (factors V, VIII, Hageman, PTA and fibrinogen). The TGT is carried out by measuring the activity of plasma thromboplastin formed from a mixture containing serum, adsorbed plasma, platelets or platelet substitute, and calcium. Since thromboplastin generates only if all four reagents are present, replacement of the patient's serum or plasma with normal serum or plasma can lead to a preliminary delineation of the patient's abnormality.

We have shown in previous reports (5, 6) that acceleration of thromboplastin formation, when present, has always been restricted to the plasmatic reagent. Serum activity in the thromboplastin generation test in four representative cases in which accelerated thromboplastin formation was exhibited is shown in Table I. Serums in Patients 1, 2 and 3 were normal while the serum in Patient 4 was actually retarded because of the administration of coumarin drugs. The thromboplastin-generating activity of the plasmatic reagent in these patients ranged from two to five times normal. Because of this observation, it seemed unlikely that the serum factors could be responsible for the acceleration process.

Platelets likewise appear to bear no direct relationship to the phenomenon of plasma acceleration, since we have observed it in thrombocytopenic

TABLE I

Serum activity in the TGT in patients with excess TGA plasma *

Incubation time	Typical control serum	Coagulation time			
		Pt. 1	Pt. 2	Pt. 3	Pt. 4
min		*sec*		*sec*	
1	65	90	93	78	52
3	47	66	68	72	48
5	45	42	47	41	45
7	32	30			47
8	21	16	26	15	
9	12	14	15	12.5	39
10	10	*12*	12	11	36
11	*9.5*		*10*	*9*	
13	11	14	11.5	10.5	32
Plasma activity in TGT, times normal		2	2	4	5

* Samples of serum (1:10) from 4 patients with thromboembolic disorders were combined with normal control plasma (1:25) in the TGT. Patient 4 was being treated with coumarin drugs; prothrombin time was 35 seconds at time of test. His serum yielded reduced thromboplastic activity; serum from the other three patients did not deviate significantly from normal.

TABLE II

*Stability of TGA at 56° C**

Incubation time	Normal control plasma	Excess TGA plasma unheated	Excess TGA plasma heated at 56° C†			
			5 min	10 min	15 min	20 min
	Clotting time					
min	*sec*		*sec*		*sec*	
1	81	56	48	76	70	108
3	66	13	17	32	25	79
5	38	*10.5*	11	12	12	42
7	26	10.5	*10.5*	*11.5*	*11.5*	23
8	14	10.5	11	12	12	16
9	*13.5*	11.5	11.5	13	12.5	*14*
10	14		12		12.5	15

* Plasma containing excess of TGA was adsorbed with $BaSO_4$ and diluted 1:50 in the TGT; normal serum was diluted 1:10. Increase in rate of generation and in amount of thromboplastin formed is evident.

† To maintain optimal AHG and factor V activity in heated samples, 1 vol fresh normal plasma (1:25) was mixed with 1 vol heated plasma (1:25); this mixture was then used as the plasmatic reagent in the TGT.

plasmas; and in our modification of the TGT, soybean phosphatide is used as a platelet substitute.

The series of experiments reported here were carried out for the purpose of determining which of the recognized factors present in adsorbed plasma and absent from serum, namely factors V, VIII and fibrinogen, might be responsible for the excessive thromboplastin formation in certain patients. Because we have not observed increased concentrations of fibrinogen in the plasma of these patients, our attack was directed primarily toward factors V and VIII. As will be shown subsequently, fibrinogen appears to be excluded as the cause of increased formation of thromboplastin in these cases.

Heat-stability studies. We have found that excess TGA activity persists after warming the plasma to 37° C for 30 minutes and to 24° C for 24 hours, and after storage at − 20° C for as long as 1 year. A comparison of its lability at 56° C with factor V and AHG was carried out.

The stability of factor V (labile factor) was measured by determining the corrective effect of normal plasma (1 vol) on the prothrombin time of aged normal plasma (9 vol), after the normal plasma had been exposed to a temperature of 56° C for various lengths of time. Normal plasma that had shortened the prothrombin time of the aged plasma from 85 to 30 seconds before heating exerted no effect on the long prothrombin time of the aged plasma after 1 minute at 56° C. We found that after 15 seconds at 56° C, 30 per cent of factor V activity remained; after 30 seconds, 12 per cent; after 45 seconds, 5 per cent; and after 60 seconds, 0 per cent. The thermolability of factor V in excess TGA plasma was indistinguishable from normal.

Stability of AHG was determined by heating fresh normal plasma to 56° C for various periods and then determining the effect of this plasma on AHG-deficient plasma by means of plasma recalcification times and thromboplastin generation tests. By these methods, AHG activity of nor-

TABLE III

Electrophoretic localization of TGA *

Incubation time	Control system	Normal plasma fractions					Excess TGA plasma fractions				
		Albumin +α_1	α_2	β	γ_1	γ_2	Albumin	α_1	α_2	β	$\gamma_1+\gamma_2$†
	Clotting time										
min	*sec*	*sec*		*sec*		*sec*		*sec*		*sec*	
1	113	73	79	78	72	100	81	81	77	61	70
3	79	72	73	65	50	72	78	86	68	52	52
5	62	52	54	53	30	53	69	65	65	20	35
7	41	36	38	22	14	42	49	35	30	*10*	13
8	25	27	23	15	12	27	37	24	17	11	12
9	18	15	16	14	12	19	21	16	14	11	*11*
10	14	13.5	15	*11*	*11.5*	*13*	15	*15*	11	12	11
11	*13.5*	*13*	*14*	11	13	13	15	15	*10.5*	12.5	12
12	14	13.5	14	12		14	*13*	15	11		13

* Barium sulfate-adsorbed normal plasma and excess TGA plasma (Patient 1, Table IV) were electrophoresed on paper strips (see Methods); zones were eluted with buffered saline and each was then used as diluent for normal plasmatic reagent in the TGT instead of the usual saline diluent. Accelerator activity was found in γ_1 and β zones, the former related to activity of AHG and factor V, the latter to TGA (see text).

† The γ_1 and γ_2 zones were not clearly separable in this plasma and consequently were tested together.

TABLE IV

Summary of electrophoretic analyses of TGA activity (in β-globulin) and AHG activity (in γ-globulin) in the plasmas of seven patients with excess TGA activity *

Pt.	β-globulin (TGA) activity				γ_1-globulin (AHG) activity			
	Normal control plasma		Excess TGA plasma		Normal control plasma		Excess TGA plasma	
	Shortest clotting time	at Incubation time	Shortest clotting time	at Incubation time	Shortest clotting time	at Incubation time	Shortest clotting time	at Incubation time
	sec	*min*	*sec*	*min*	*sec*	*min*	*sec*	*min*
1	11	10	10	7	11.5	10	11	9
2	12	9	12	8	12	8	12.5	8
3	12.5	8	9.5	8	11.5	8	11	8
4	12.5	11	11	11	11	9	10	9
5	13.5	8	11	7	14	8	14	8
6	14	10	12	9	13	9	14	9
7	14.5	16	13	13	17	16	16.5	16

* The TGT was performed as described in Table III. Only the shortest clotting times and the time of incubation required to reach these times are listed. The AHG activity in all cases was comparable between the plasmas of patients and controls. In every instance there was more accelerating activity in the β-globulin of the excess TGA plasma than in the β-globulin of the control plasma subjected to electrophoresis concurrently.

mal plasma was found to be depressed about 70 per cent in 1 minute and about 90 per cent in 5 minutes, and was undetectable after 10 minutes of exposure to 56° C. The thermolability of AHG in excess TGA plasma was indistinguishable from normal.

The effect of heating excess TGA plasma to 56° C is shown in Table II. It can be seen that the rate of thromboplastin generation was little affected for the first 15 minutes during which time both factor V and AHG activities had completely disappeared.

Electrophoretic identification. Fresh samples of seven different barium sulfate-adsorbed excess TGA plasmas, along with seven samples of normal control plasma similarly treated, were subjected to paper electrophoresis. Eluates of various zones on the paper strips were used as diluents for the normal plasmatic reagent in the TGT. Adsorbed plasma was used for electrophoresis in order to simplify the analyses by prior removal of factors VII, IX, X and prothrombin.

The complete TGT's of a typical case (Patient 1, Table IV) are listed in Table III. All seven cases are summarized in Table IV. Increased thromboplastin formation was obtained when eluates from β and γ_1 fractions of either the normal control or excess TGA plasma were used as diluents. The γ_1 eluates of both the control and the excess TGA plasma showed approximately the same activity; however, the β fraction of the excess TGA plasma exhibited a greater degree of thromboplastic activity than did the corresponding β fraction from normal plasma. Comparable results were obtained invariably in each of the other six studies (Table IV). It seems clear that TGA and AHG can be differentiated electrophoretically.

Eluates from the various fractions of both normal plasma and excess TGA plasma were tested for AHG activity (Table V). In every instance the eluate from the γ_1 region decreased the recalcification time of AHG-deficient plasma, while eluates from the other fractions failed to influence significantly the clotting time of the hemophilic plasma. The apparent difference between our finding and that of Lewis, Walters, Didisheim and Merchant (15) may be technical, since this group used continuous flow electrophoresis.

TABLE V

AHG activity in electrophoretic fractions of normal plasma *

Plasma	Clotting time
	sec
AHG-deficient plasma	440
AHG-deficient plasma plus:	
Albumin (fresh plasma)	450
β (fresh plasma)	400
γ (fresh plasma)	300
γ_1 (aged plasma)	410

* Eluates of paper electrophoretic fractions were added to fresh AHG-deficient plasma in 1:10 ratio; plasma clotting times were determined on mixtures. Sample of aged plasma (free of AHG) was similarly treated as control. AHG activity seemed to concentrate in γ_1 zone.

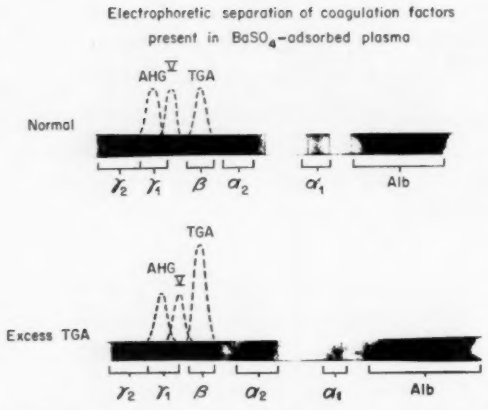


FIG. 1. COAGULATION ACTIVITIES IN THE β AND γ ZONES OF NORMAL PLASMA AND EXCESS TGA PLASMA, BOTH ADSORBED WITH $BaSO_4$ BEFORE PAPER ELECTROPHORESIS.

To exclude the effect of factor V, which usually travels with the γ_1 plasma proteins (14), the eluates of the β fraction of both the control plasma and the excess TGA plasma were heated to 56° C for 1 minute to inactivate completely any factor V that might have been present. These heated samples were then used as diluents for the plasmatic reagent in the TGT, as above. Neither the eluate from the control nor that from the excess TGA plasma showed any loss of activity after heating. It is assumed, therefore, that there was virtually no factor V activity in the β fractions, and in turn that the β-globulin clotting activity was not attributable to factor V. In each electrophoretic test, fibrinogen was heavily concentrated at the point of application of the plasma to the paper strips in the γ region, and far removed from the β fraction.

Hageman factor activity has been shown to reside in the γ_1 fraction (16), and PTA activity in the β-γ interspace (17); unlike TGA, however, both are present in serum.

Electrophoretic analyses of barium sulfate-adsorbed serum (lacking prothrombin and factors V, VII, VIII, IX, X and TGA, but containing PTA and Hageman factor) from normal controls and from patients with accelerated clotting were carried out and eluates from the various fractions were studied in a manner similar to that used for the plasma. No evidence of increased thromboplastic activity was found when these eluates were used as diluents in the TGT. It was assumed from this experiment that the presence of excessive amounts of Hageman factor and PTA in the TGT do not materially influence thromboplastin generation. The electrophoretic zones of activity of TGA, factor V, and AHG in normal and excess TGA plasma are illustrated in Figure 1.

Precipitating with ammonium sulfate. To corroborate the findings obtained by electrophoresis, precipitation experiments with ammonium sulfate

TABLE VI

Localization of TGA by precipitation with ammonium sulfate *

Incubation time	Control system†	Ammonium sulfate saturation: Fractions of normal plasma 25%	25–35%	35–40%	40–45%	Excess TGA plasma fractions 25%	25–35%	35–40%	40–45%
		Clotting time							
min	*sec*	*sec*		*sec*		*sec*		*sec*	
1	78	69	65	66	79	64	76	74	66
3	74	49	49	41	64	55	96	53	66
5	62	19	19	19	40	37	70	19	39
7	35	*11*	14	12	16	17	38	11	
8	22	11	14	14	13.5	12.5	17	10	16
9	14	12	13	*11.5*	14.5	*12*	15	*8.5*	*13*
10	*13*	12	*12*	11.5	*13*	14	*12.5*	9.5	14
11	13	12	12.5	13	14		14		14
12	14	13.5		14	14		14	13.5	

* Barium sulfate-adsorbed normal plasma and excess TGA plasma were fractionated with various concentrations of saturated ammonium sulfate. Precipitates were dissolved, freed of $(NH_4)_2SO_4$ by dialysis, and used as diluents (1:50) for plasmatic reagent (normal plasma) in the TGT. AHG activity is evident in 25% fractions, and TGA in 35 to 40% fractions, particularly in excess TGA plasma (see text). Reduced AHG activity of excess TGA plasma is probably related to 3-month storage of this plasma at −20° C.

† Buffered saline used as diluent.

were performed. In these procedures, fresh normal plasma (control) and excess TGA plasma were initially adsorbed with barium sulfate and then subjected to fractionation with saturated ammonium sulfate. The precipitates from the various concentrations were dissolved, dialyzed and then used as diluents in the TGT. The influence of these fractions on the retarded TGT is shown in Table VI.

Both the 25 per cent and the 35 to 40 per cent ammonium sulfate saturation fractions of normal plasma and the excess TGA plasma exhibited increased thromboplastic activity. When tested against AHG-deficient plasma, the 25 per cent fractions were the most active in correcting the long clotting time of the hemophilic plasma; there was little difference between the 25 per cent fraction derived from normal plasma and that from excess TGA plasma (Table VII). On the other hand, the 35 to 40 per cent fraction from excess TGA plasma was significantly more active in accelerating normal thromboplastin generation in the TGT than was the same fraction from normal plasma. Neither 35 to 40 per cent fraction exhibited any AHG activity.

Dialysis studies. Fresh excess TGA plasma, barium sulfate-adsorbed excess TGA plasma and the 35 to 40 per cent ammonium sulfate saturation fraction of excess TGA plasma were dialyzed in cellophane bags against 0.9 per cent sodium chloride for 20 hours at 4° C. No reduction of TGA activity was found in these plasmas; this is compatible with the concept that TGA is a protein.

TABLE VII

AHG activity in fractions obtained by precipitation from normal plasma with ammonium sulfate *

	Clotting time
	sec
AHG-deficient plasma	209
AHG-deficient plasma plus fraction obtained at saturation of:	
25%	157
25–35%	190
35–40%	204
40–45%	211
45–50%	196

* Influence of ammonium sulfate-precipitated fractions of fresh normal plasma on prolonged recalcification time of AHG-deficient plasma is shown. Percentages refer to strength of saturated ammonium sulfate solution at which precipitation occurred. Precipitates were reconstituted after dialysis to equal plasma concentrations, then were added to AHG-deficient plasma in 1:9 ratio, and plasma clotting times determined on mixtures. The 25 per cent fractions presumably contained AHG (see text).

Effect of adsorbent chemicals. Each milliliter of plasma containing excess TGA was treated with barium sulfate (50 mg) for 5 minutes at 24° C, tricalcium phosphate (0.1 ml of a 0.2 M suspension) for 5 minutes at 24° C, or aluminum hydroxide (0.1 ml of a 6.1 per cent suspension) at 0° C for varying periods of time and then tested in the TGT. Normal plasma similarly treated was used as the control. The results are shown in Table VIII. Accelerated generation of thromboplastin was equally apparent after the barium sulfate and tricalcium phosphate treatment. On

TABLE VIII

Action of adsorbing agents on TGA *

Incubation time	$BaSO_4$-adsorbed		$Ca_3(PO_4)_2$-adsorbed		$Al(OH)_3$-adsorbed†	
	Normal plasma	Excess TGA plasma	Normal plasma	Excess TGA plasma	Normal plasma	Excess TGA plasma
	Clotting time					
min	*sec*		*sec*		*sec*	
1	73	83	63	63	70	77
3	69	50	59	62	71	69
5	42	45	49	47	50	52
7	32	26	37	31	35	36
9	19.5	13	21	16	21	22
10	16	12	17	14	17	19
11	*14.5*	*11.5*	*16*	*12.5*	*15.5*	*16*
12	15	12	16	12.5	15.5	16
13			17	13	16.5	17

* Normal plasma and excess TGA plasma were treated with $BaSO_4$, $Ca_3(PO_4)_2$ or $Al(OH)_3$ and tested in the retarded TGT. Accelerated generation of thromboplastin was not evident after adsorption with $Al(OH)_3$.

† Adsorbed for 5 minutes.

TABLE IX

Effect of prolonged $Al(OH)_3$ adsorption on TGA content of normal plasma *

Incubation time	$BaSO_4$-treated plasma	$Al(OH)_3$-treated plasma		
		15 min	30 min	45 min
	Clotting time			
min	*sec*		*sec*	
1	80	66	68	78
3	64	65		
5	54	50	61	60
7	30	31		
8	19	26	46	54
9	12	18	33	46
10	10			
11	*9.5*	15.5	21	29
12			19	21
13	10	15	16	18
15	11	*14.5*	*15.5*	*17*
17		16	16	18

* Samples of fresh normal plasma were adsorbed with $BaSO_4$ (control) and with $Al(OH)_3$ for 15, 30 and 45 minutes. Activity of each treated sample was measured in the TGT using normal serum diluted 1:10 in buffered saline. All samples were diluted 1:25 with buffered saline containing adsorbed normal serum as source of optimal PTA and Hageman factor. Generation of thromboplastin diminished as exposure to $Al(OH)_3$ increased.

the other hand, treatment with aluminum hydroxide for 5 minutes sharply reduced the excessive TGA activity.

In order to determine the effect of prolonged aluminum hydroxide adsorption on normal plasma, samples of plasma were exposed to aluminum hydroxide for periods of 15, 30 and 45 minutes and were then tested in the TGT (Table IX). Thromboplastin-generating activity diminished steadily the longer the plasma remained in contact with the aluminum hydroxide. In order to rule out the possibility that the aluminum hydroxide was adsorbing PTA or Hageman factor, barium sulfate-adsorbed normal serum was added to the TGT. Since PTA and Hageman factors are present in such serum, the diminished thromboplastin generation cannot be attributed to them.

Theoretically, the results of adsorption with aluminum hydroxide could be attributed to removal of factor V, AHG or TGA from the plasma. To determine the effect of prolonged aluminum hydroxide adsorption on factor V activity, 1 vol of normal plasma, treated for 15, 30 and 45 minutes, was added to 9 vol of aged plasma, and one-stage prothrombin times were determined on the mixtures; the prothrombin time of the aged plasma alone was 65 seconds. All of the samples shortened the prothrombin time to 36 to 38 seconds. Since fresh, unadsorbed normal plasma shortened the prothrombin time of the aged plasma to the same range, namely 37.5 seconds, it was concluded that factor V activity was not affected by prolonged adsorption with aluminum hydroxide.

The effect of aluminum hydroxide on AHG activity of normal plasma was evaluated by measuring the effect of such treated plasma on the prolonged clotting time of AHG-deficient plasma. Samples of the treated plasma were added to the hemophilic plasma in a 1:9 proportion. It was found (Table X) that AHG activity was not re-

TABLE X

Effect of prolonged $Al(OH)_3$ adsorption on AHG activity of normal plasma *

Sample	Plasma clotting time	Added AHG, % of normal	Estimated AHG, action, % of normal
	sec		
AHG-deficient plasma	480	0	
AHG-deficient plasma plus:			
Normal plasma untreated	135	100	
Normal plasma treated for:			
10 min	140		90
30 min	142		85
45 min	137		95
Normal plasma untreated and diluted 1:4 in saline	170	25	
Normal plasma treated for 45 min and diluted 1:4 in saline	170		25

* Fresh normal plasma before and after adsorption with $Al(OH)_3$ for 10, 30 and 45 minutes was mixed with AHG-deficient plasma and effect on clotting time noted. Plasmas were mixed in proportion of 9 parts AHG-deficient plasma to 1 part test plasma. There was no obvious effect of $Al(OH)_3$ on AHG of normal plasma.

TABLE XI

Influence of $Al(OH)_3$ on normal plasma and excess TGA plasma in correction of defect of hemophilic plasma *

Fraction of normal or excess TGA plasma in mixtures	$BaSO_4$-adsorbed					$Al(OH)_3$-adsorbed†				
	Normal plasma		Excess TGA plasma			Normal plasma		Excess TGA plasma		
	Clotting time	Activity based on dilution	Clotting time	Activity‡	Rel. act.	Clotting time	Activity‡	Clotting time	Activity‡	Rel. act.
	sec	%	*sec*	%		*sec*	%	*sec*	%	
1/10	123	100	104							
1/20	139	50	110			143	45	123	100	2.2
1/40	155	25	127	90	3.6	151	30	142	45	1.5
1/80	165	12.5	144	45	3.6	162	14	156	25	1.8
1/160	184	6.3	150	30	4.8					
1/320	210	3.1	170	10	3.3					

* $BaSO_4$ and $Al(OH)_3$-adsorbed samples of normal and excess TGA plasma were added in various proportions to AHG-deficient plasma and clotting times were determined on mixtures. Clotting time of AHG-deficient plasma alone was 390 seconds. Relative activity (Rel. act.) was calculated from: excess TGA % activity/normal % activity. AHG activity of normal plasma was the same whether adsorbed in $BaSO_4$ or $Al(OH)_3$. Apparent AHG activity was 3.8 times normal when excess TGA plasma was adsorbed with $BaSO_4$; half this increased activity disappeared after $Al(OH)_3$ adsorption, suggesting TGA rather than AHG effect (see text).

† Plasma adsorbed for 10 minutes.

‡ Compared with $BaSO_4$-adsorbed normal plasma.

duced by 10, 30 or 45 minutes of exposure of normal plasma to aluminum hydroxide.

Since aluminum hydroxide had no apparent effect on the AHG activity of normal plasma, but did on the TGA activity of excess TGA plasma, the effect of this adsorbent on the AHG activity of excess TGA plasma was studied, barium sulfate adsorption being used as a control. Samples of adsorbed normal and excess TGA plasma were added in decreasing concentrations to hemophilic plasma; plasma clotting times were determined on the mixtures. These results are shown in Table XI. After barium sulfate adsorption the excess TGA plasma exerted about four times the activity of normal plasma in correcting the prolonged clotting time of the AHG-deficient plasma. After adsorption for 10 minutes with aluminum hydroxide, the activity was reduced to about twice normal. Treatment with aluminum hydroxide did not apparently alter the corrective ability of the normal plasma, however. It was assumed from this and the previous experiment (Table X) that the increased activity of the excess TGA plasma in correcting the clotting time of the hemophilic plasma was attributable to the accelerator effect of TGA and not to increased AHG, since AHG is apparently not adsorbed by aluminum hydroxide. The similarity of action of TGA and AHG suggests that the positions of both factors in the scheme of blood coagulation are closely allied.

Prothrombin time of diluted plasma. Thus far, the evidence places TGA activity early in the clotting reaction (prothromboplastic step), because of acceleration in the TGT associated with normal prothrombin times. In order to assess any possible second-stage activity of TGA, prothrombin times, which are independent of the first stage, were determined on diluted specimens of a number of excess TGA plasmas (Table XII). The results tend to exclude any second-stage activity comparable to that exhibited by factors V, VII and X. The indirect implication is that excess TGA activity is not the result of excess factor V.

TABLE XII

Prothrombin time of undiluted and diluted normal plasma and four samples of excess TGA plasma *

Plasma dilution	Prothrombin time				
	Normal plasma	Excess TGA plasma samples			
		No. 1	No. 2	No. 3	No. 4
	sec	*sec*	*sec*	*sec*	*sec*
Undiluted	17–19	19	17	18.8	18
1:5	30–49	37.5	37	35	41
1:10	56–67	58	49	57	61
Acceleration of thromboplastin formation in TGT times normal	Normal	3	3	3	2

* There was no obvious effect of excessive TGA on second step of clotting (thromboplastin activation or cothromboplastic step).

TABLE XIII

Estimation of factor V activity in excess TGA plasma *

Proportion of normal plasma of "excess TGA plasma" to aged plasma	Prothrombin time				
	Normal plasma values, range	Excess TGA plasma samples			
		No. 1	No. 2	No. 3	No. 4
	sec	*sec*		*sec*	
1:10	38–46	40	30	40	43
1:20	48–56	49	43	48	50
Plasma acceleration in TGT, times normal		4	3	2	2

* Factor V activity in 4 samples of excess TGA plasma was determined by adding this plasma to aged normal plasma in proportions of 1:10 and 1:20 and determining one-stage prothrombin time on mixtures. Prothrombin time of aged plasma was 85 seconds. Only plasma no. 2 exhibited any suggestion of excessive factor V activity.

Determination of factor V activity in excess TGA plasma. Factor V activity in excess TGA plasma was assessed directly by noting the degree of correction of the prolonged prothrombin time of aged plasma on mixing the two plasmas. Three of the four studied showed no evidence of increased activity of factor V; in the fourth instance, there was modest acceleration as compared to control values (Table XIII).

Because of this one apparent exception to an otherwise sharp differentiation of TGA and factor V, further discrimination was sought by way of the TGT (Table XIV). Excess TGA plasma, which had approximately three times the normal thromboplastic activity in the TGT when measured in the conventional manner, was heated for 2 minutes at 56° C to inactivate factor V completely. When tested in the TGT this plasma still exhibited approximately the same degree of excess activity as before heating. (A constant source of factor V was added in the form of fresh AHG-deficient plasma.) Since increased thromboplastic activity persisted in the excess TGA plasma despite complete inactivation of its factor V content, it was assumed that factor V was not responsible for the increased activity of the unheated plasma in the TGT.

Effect of coumarin drug therapy. Many of the patients with thrombotic arterial and venous disease whose plasma showed the phenomenon of acceleration in the TGT were treated with one or another coumarin drug. In spite of lengthened prothrombin time and retardation of the serum activity in the TGT, their plasmas showed no reduction in excessive TGA activity. Results of the TGT of the plasma from one such patient on long-term anticoagulant therapy are illustrated in Figure 2. This patient's prothrombin time was effectively prolonged by drug therapy, but the TGA activity of his plasma remained persistently elevated. We have also observed excess TGA in the plasma of certain patients with thrombotic disease in the presence of long-standing hepatic disease with demonstrable deficiencies of factors V, VII, X and prothrombin.

DISCUSSION

By retarding the conventional thromboplastin generation test (retarded TGT), we have been able to demonstrate an unusually rapid formation of plasmatic thromboplastin in 75 per cent of 52 persons with acute peripheral arterial thrombosis complicating arteriosclerosis obliterans (18) and in 50 per cent of 40 patients with idiopathic thrombophlebitis. The present study provides a detailed comparison of this accelerated clotting activity with each of the recognized blood clotting

TABLE XIV

Effect of heating excess TGA plasma for 2 minutes at 56° C on accelerated thromboplastin generation in TGT *

Incubation time	Control system†	Excess TGA system‡
min		
1	54	54
3	56	53
5	36	32
7	23	20
8	18.5	15
9	18	12
10	17	12
11	16	11
12	16	10.5
14	17	11.5

* Factor V activity was removed from $BaSO_4$-adsorbed excessTGA plasma by heating at 56° C for 2 minutes. Mixture of equal parts of this plasma and fresh unheated $BaSO_4$-adsorbed AHG-deficient plasma (each diluted 1:25) constituted plasmatic reagent for TGT. Final concentration of factor V was 1:50 (all from AHG-deficient plasma), and of AHG 1:50 (all from heated plasma). Normal plasma was similarly treated. TGA activity was not appreciably reduced by heating despite complete destruction of factor V.

† Normal plasma heated to 56° C for 2 minutes (1:25) plus fresh AHG-deficient plasma (1:25).

‡ Excess TGA plasma (exhibiting approximately 3 times normal activity in thromboplastin formation when tested in TGT prior to heating) heated to 56° C for 2 minutes (1:25) plus fresh AHG-deficient plasma (1:25).

factors. This has led us to the conclusion that the rapid generation of thromboplastin in these patients cannot be satisfactorily explained on the basis of any currently identified blood-clotting factor. For clarification, we have applied to the newly recognized hypercoagulable activity the name "thromboplastin generation accelerator" (TGA), since its function is thereby described.

TGA was found to migrate electrophoretically with the β-globulins of plasma, and in this regard is like factor VII (14) and factor IX (19). TGA, like AHG (factor VIII), labile factor (V), Hageman factor, PTA, and fibrinogen, was not adsorbed from plasma by barium sulfate. TGA did not survive clotting, and is therefore absent from serum, like antihemophilic globulin (factor VIII), labile factor (V), prothrombin and fibrinogen. On the basis of these simple experiments alone, there can be only a few well authenticated coagulation factors that might have the TGA activity.

Factor V. TGA is considerably more heat stable than factor V. Within 1 minute at 56° C, factor V activity in human plasma was completely dissipated, while TGA remained active for at least 15 minutes. When paper electrophoresis of fresh plasma was carried out, factor V activity could be demonstrated in the β-γ interspace (14), while TGA activity was confined to the β fraction. Prolonged contact with aluminum hydroxide decreased TGA activity in direct proportion to the length of exposure. In contrast, factor V activity remained unaffected even after 45 minutes of adsorption. When assays of factor V were carried out on plasmas containing excessive amounts of TGA, a moderate increase was found in only one of the four studied.

Antihemophilic globulin (AHG, factor VIII). TGA was found to be somewhat more stable at 56° C than AHG, which was completely inactivated within 10 minutes. The stability of AHG, therefore, lies midway between that of factor V and that of TGA.

After electrophoresis of fresh barium sulfate-adsorbed plasma, maximal AHG activity could be demonstrated in the γ_1-globulin fraction, but not in the β-globulin zone of either normal plasma or plasma containing excess TGA. After precipitation with ammonium sulfate, AHG activity was confined to the 25 per cent saturation fraction (euglobulin) while TGA was present in the 35 to 40 per cent fraction (pseudoglobulin). Another distinction was the inability of aluminum hydroxide to adsorb AHG; after prolonged exposure, most or all of the TGA was adsorbed.

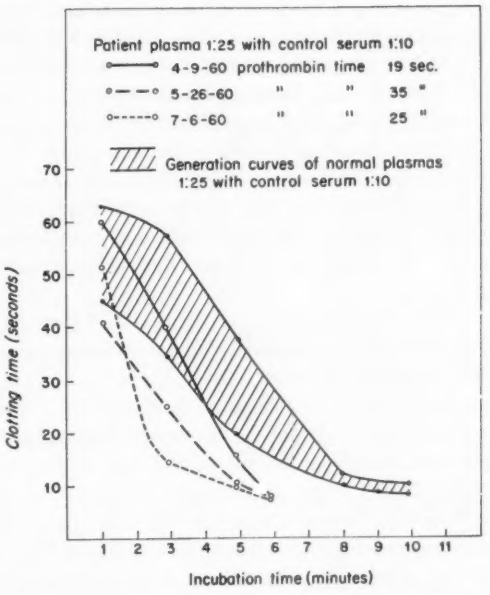


FIG. 2. EFFECT OF COUMARIN ANTICOAGULANT THERAPY ON TGA ACTIVITY. This 54 year old man had noted intermittent claudication in right calf for 5 years. On March 8, 1960, he sustained a sudden aortoiliac occlusion. An aortofemoral bypass graft (Teflon) inserted on April 1, 1960, resulted in return of normal arterial pulsations to both lower extremities. Anticoagulant therapy with Warfarin sodium was instituted on April 26, 1960, with plans to continue it on long-term basis. TGA activity remained essentially unchanged during a 3 month period, despite continuous and adequate therapy with Warfarin sodium.

Fibrinogen. Fibrinogen is closely associated with AHG in the γ_1 electrophoretic and 25 per cent ammonium sulfate saturation fractions; like AHG it was cleanly separated from the zone occupied by TGA.

PTA and Hageman factor. Since both PTA and Hageman factor were present in barium sulfate-adsorbed plasma, the question might arise concerning the part played by these substances in the hypercoagulable state. The fact that both PTA and Hageman factor are present in an active state in serum (16, 17) would seem to distinguish them from TGA, since the thromboplas-

tin-generating activity of serum from none of our patients with thromboembolism has been hyperactive. Furthermore, eluates of electrophoretically processed normal, barium sulfate-adsorbed serum, containing only PTA and Hageman factor as far as is known, did not enhance thromboplastin formation in a TGT of normal plasma and serum.

Both PTA, in the β-γ interspace, and Hageman factor, in the γ_1 zone, were effectively separated from TGA, in the β zone, by electrophoresis (16, 17).

Does TGA activity correspond to previously described blood coagulation factors? Increase in concentrations of factor VII (20), factor V (21), and AHG (22) in certain clinical states has been reported but does not seem pertinent in this discussion because of the significant differences between these clotting factors and TGA.

Wessler, Reimer and Deykin (4, 23) have described a serum activity capable of inducing thrombosis. They have entitled it serum thrombotic accelerator (STA). Since STA is present in serum and is readily adsorbed by barium sulfate, it cannot be reconciled with the properties of TGA.

The absence of TGA in serum, its resistance to barium sulfate adsorption, and its greater heat stability separate it from the prephase accelerator described by Fisch (24).

Waaler (25) has reported that there is an "activation product" resulting from the interaction of PTA and Hageman factor. Certain properties of activation product and TGA are similar: electrophoretic mobility, property of being unaffected by coumarin drugs, and susceptibility to ammonium sulfate precipitation. However, Waaler found activation product reduced four-fifths by heating plasma for 30 minutes at 37° C, whereas TGA is unaltered by this same treatment. Furthermore, Waaler states that "activation product was unstable when in plasma" and "in serum a great amount of activation product was found." Neither of these descriptions is compatible with our observations of TGA activity. We are unable to find in the literature any description of a coagulation activity like that of TGA.

Function of TGA. TGA appeared to influence primarily the rate of formation, and possibly the yield, of plasma thromboplastin. In the TGT, peak thromboplastic activity was reached in one-half to two-thirds the time required by normal plasma. In addition, the activity of the thromboplastin produced was usually greater than that of the control, as evidenced by shorter clotting times in the substrate plasma. When TGA was partially removed from excess TGA plasma by adsorption with aluminum hydroxide, both the rate of formation and the amount of thromboplastin formed were reduced.

If TGA's function lies in the generation of thromboplastin, it might act in the initial prothromboplastic step, in the second or activation step, or both. These two steps can be distinguished by the prothrombin time test of Quick (7), since the addition of an extract of brain to plasma apparently results in complete bypass of the prothromboplastic step with all the factors involved. Prothrombin times were determined in the usual manner, as well as with five- and tenfold dilutions of plasma in order to detect any minute shortening of the prothrombin times. In both undiluted and diluted plasmas, prothrombin times of excess TGA plasmas were uniformly within normal limits. This would seem to isolate TGA's position in the scheme of clotting to the initial step, in association with platelets, Hageman factor, AHG, PTC and PTA.

Routine detection of excess plasmatic TGA. The original description of the TGT by Biggs and Douglas included adsorption of the plasmatic reagent with aluminum hydroxide (10); since this reagent adsorbs TGA, it is possible that those who use this technic may be unable to detect all of the TGA activity. Furthermore, the Biggs-Douglas test is carried out with the adsorbed plasma diluted only fivefold, so that the normal endpoint is reached in 2 or 3 minutes. In the Duckert modification (11) of the TGT, plasma is adsorbed with barium sulfate and is then diluted 25-fold. TGA is not adsorbed and excessive amounts may be detected because slight acceleration over the normal reaction rate may be perceptible. In our experience, an even greater dilution of the plasma (1:50) is needed to recognize accelerated thromboplastin generation unequivocally.

To date, our studies have been carried out largely in patients with arteriosclerosis obliterans or idiopathic thrombophlebitis. Because of the nature of our selection of patients, excess TGA

TABLE XV

Distinguishing properties of TGA

Coagulation factor	Present in:* Plasma	$BaSO_4$-adsorbed plasma	$Al(OH)_3$-adsorbed plasma	Serum	Lability at 56° C, grade†	Electrophoretic localization	Effect of coumarin drug therapy‡
TGA	+	+	0	0	2	β	0
AHG	+	+	+	0	3	γ_1	0
Factor V	+	+	+	0	4	β-γ (14)	0
PTC (IX)	+	0	0	+	1	β (19)	+
Factor VII	+	0	0	+	1	β (14)	+
Factor X	+	0	0	+	3 (27)	α (26)	+
Prothrombin	+	0	0	0		α_2 (14)	+
PTA	+	+	+	+	1 (28)	β-γ (17)	0
Hageman factor	+	+	+	+	1 (28)	γ_1 (16)	0
Fibrinogen	+	+	+	0		Point of appl. (14)	0
Platelets	+	0	0	0		Point of appl.	0

* Key: + = present; 0 = absent.
† Graded on a basis of 0 to 4, 0 representing nonlability, and 4 greatest lability.
‡ Key: + = depressed; 0 = no depression.

activity was usually found to be associated with recent thrombosis. Before it is concluded that the excess TGA activity is simply the result of the thrombosis, it may be recalled that normal plasma exhibits a modest coagulative activity in the β-globulin zone indistinguishable from TGA. Furthermore, in one patient studied serially for 3 years and in whom there was no further clinical evidence of thrombosis, excess TGA activity persisted unaltered. It should be stated that in one patient the excess TGA activity disappeared within 3 weeks.

Additional evidence that TGA is not prothrombin, or factor VII, IX or X, all of which tend to be depressed by coumarin-type therapy, is the fact that excess TGA activity is unaltered by such therapy. Since thrombi tend to disappear during anticoagulant therapy, the persistence of excess TGA activity again raises the question of any direct relationship between excessive TGA and thrombosis. The distinguishing properties of TGA are summarized in Table XV.

Deficiency of TGA. As with all of the other known coagulation factors, it is to be expected that deficiency states of TGA will be discovered. Unfortunately, it is not possible to distinguish a deficiency of TGA from a deficiency of AHG by any currently available TGT. Deficiency of either TGA or AHG is to be suspected when the generation of thromboplastin from the patient's plasma is limited, but is normal from the patient's serum. Deficiency of AHG could be distinguished from that of TGA by adding aluminum hydroxide-adsorbed normal plasma to the TGT, since this would contain AHG but not TGA; if such a mixture should lead to a normal generation of thromboplastin, deficiency of AHG would be confirmed. Since such a distinction has not been made in the past, it is possible that certain patients believed to have "classic hemophilia" may indeed suffer from deficiency of TGA. Perhaps with a differentiating test available, a re-evaluation of women with apparent AHG deficiency, as in vascular hemophilia or thrombasthenic hemophilia (12), should be carried out.

SUMMARY

We have described a new component of the coagulation mechanism. This substance, which we have named thromboplastin generation accelerator, or TGA, appears to be responsible for the accelerated formation of plasma thromboplastin observed in certain patients with intravascular thrombosis. It is a moderately heat-labile protein associated with the β-globulins. It is present in fresh plasma but not in serum. Its site of action seems to be the initial stage of clotting, where it appears to influence primarily the rate of formation, and possibly the yield, of plasma thromboplastin. It is unaffected by therapy with coumarin drugs.

REFERENCES

1. Mustard, J. F. Blood coagulation in subjects with and without clinical evidence of atherosclerotic vessel disease. Canad. med. Ass. J. 1958, 79, 554.

2. Sharnoff, J. G. Increased pulmonary megakaryocytes —probable role in postoperative thromboembolism. J. Amer. med. Ass. 1959, **169**, 688.
3. McDonald, L., and Edgill, M. Changes in coagulability of the blood during various phases of ischaemic heart-disease. Lancet 1959, **1**, 1115.
4. Wessler, S., and Reimer, S. M. The role of human coagulation factors in serum-induced thrombosis. J. clin. Invest. 1960, **39**, 262.
5. Owen, C. A., Jr., and Thompson, J. H., Jr. Thrombosis from the viewpoint of a blood coagulationist *in* Pathogenesis and Treatment of Cerebrovascular Disease, W. S. Fields, Ed. Springfield, Ill., Charles C Thomas, 1961, pp. 105–125.
6. Spittel, J. A., Jr., Pascuzzi, C. A., Thompson, J. H., Jr., and Owen, C. A., Jr. Acceleration of early stages of coagulation in certain patients with occlusive arterial or venous diseases: Use of a modified thromboplastin generation test to evaluate clot acceleration. Proc. Mayo Clin. 1960, **35**, 37.
7. Quick, A. J. On the quantitative estimation of prothrombin. Amer. J. clin. Path. 1945, **15**, 560.
8. Hurn, M., Barker, N. W., and Magath, T. B. The determination of prothrombin time following the administration of Dicumarol, 3,3′-methylenebis (4-hydroxycoumarin), with special reference to thromboplastin. J. Lab. clin. Med. 1945, **30**, 432.
9. Owen, C. A., Jr., Mann, F. D., Hurn, M. M., and Stickney, J. M. Evaluation of disorders of blood coagulation in the clinical laboratory. Amer. J. clin. Path. 1955, **25**, 1417.
10. Biggs, R., and Douglas, A. S. The thromboplastin generation test. J. clin. Path. 1953, **6**, 23.
11. Duckert, F., Flückiger, P., Isenschmid, H., Matter, M., Vogel-Meng, J., and Koller, F. A modification of the thromboplastin generation test: Role of calcium, factor V and factor VII in prothrombin conversion initiated by active blood thromboplastin. Acta haemat. (Basel) 1954, **12**, 197.
12. Owen, C. A., Jr., and Thompson, J. H., Jr. Soybean phosphatides in prothrombin-consumption and thromboplastin-generation tests: Their use in recognizing "thrombasthenic hemophilia." Amer. J. clin. Path. 1960, **33**, 197.
13. Mertz, E. T., and Owen, C. A. Imidazole buffer: Its use in blood clotting studies. Proc. Soc. exp. Biol. (N. Y.) 1940, **43**, 204.
14. Owen, C. A., Jr., and McKenzie, B. F. Application of paper electrophoresis to separation of blood-clotting factors. J. appl. Physiol. 1954, **6**, 696.
15. Lewis, J. H., Walters, D., Didisheim, P., and Merchant, W. R. Application of continuous flow electrophoresis to the study of the blood coagulation proteins and the fibrinolytic enzyme system. 1. Normal human materials. J. clin. Invest. 1958, **37**, 1323.
16. Thompson, J. H., Jr., Spittel, J. A., Jr., Pascuzzi, C. A., and Owen, C. A., Jr. Laboratory and genetic observations in another family with the Hageman trait. Proc. Mayo Clin. 1960, **35**, 421.
17. Rosenthal, R. L. Properties of plasma thromboplastin antecedent (PTA) in relation to blood coagulation. J. Lab. clin. Med. 1955, **45**, 123.
18. Spittel, J. A., Jr., Pascuzzi, C. A., Thompson, J. H., Jr., and Owen, C. A., Jr. Accelerated thromboplastin generation in patients with acute peripheral arterial occlusion complicating arteriosclerosis obliterans. Unpublished data.
19. Aggeler, P. M., Spaet, T. H., and Emery, B. E. Purification of plasma thromboplastin factor B (plasma thromboplastin component) and its identification as a beta$_2$ globulin. Science 1954, **119**, 806.
20. Loeliger, A., and Koller, F. Behaviour of factor VII and prothrombin in late pregnancy and in the newborn. Acta haemat. (Basel) 1952, **7**, 157.
21. Forwell, G. D., and Ingram, G. I. C. The effect of adrenaline infusion on human blood coagulation. J. Physiol. (Lond.) 1957, **135**, 371.
22. Pitney, W. R., and Elliott, M. H. Plasma antihaemophilic-factor concentrations in the Australian aborigine and in conditions associated with hyperglobulinaemia. Nature (Lond.) 1960, **185**, 397.
23. Reimer, S. M., Wessler, S., and Deykin, D. Serum thrombotic accelerator (STA) activity: Its relation to the first phase of clotting. Proc. Soc. exp. Biol. (N. Y.) 1960, **105**, 438.
24. Fisch, U. Über einen neuen Accelerator der Blutthrombokinasebildung. Thromb. Diath. haemor. 1958, **2**, 60.
25. Waaler, B. A. Contact activation in the intrinsic blood clotting system. Scand. J. clin. Lab. Invest. 1959, **11**, suppl. 37.
26. Duckert, F. On the properties of the Stuart-Prower factor *in* New Blood Clotting Factors, I. S. Wright, F. Koller and F. Streuli, Eds. Stuttgart, Friedrich-Karl Schattauer-Verlag, 1959, pp. 32–36.
27. Graham, J. B. Stuart clotting defect and Stuart factor. *Ibid.*, pp. 22–31.
28. Soulier, J. P. Our present knowledge on PTA factor. *Ibid.*, pp. 123–131.

PROCEEDINGS OF THE FIFTY-THIRD ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, INC., HELD IN ATLANTIC CITY, N. J., MAY 1, 1961

PRESIDENTIAL ADDRESS

THE SHAPE OF CLINICAL INVESTIGATION

BY CLEMENT A. FINCH

It is customary and it seems to me appropriate for the President of our Society to devote his address to some general aspect of clinical investigation with which our Society is concerned. Past presidents have described changes which have occurred in contemporary medicine and research. I intend to speak briefly on the same theme; and if what I have to say should have scant merit, it shall at least have this virtue: that it was a footnote to what had been said by my capable predecessors.

However, a word first about the basic nature and needs of clinical investigation, since these are not subject to change. There is first the individual of keen mind and dedicated purpose and second, an environment in which such a person may work with a minimum of coercion and prejudice and a maximum of inspiration. These needs have been served best in the great universities, where creative scholarship has been catalyzed through associations of men of many academic disciplines engaged in the pursuit of truth. Such spawning beds as these for the clinical investigator must be considered inviolate. If we are agreed that these are the fixed requirements in our discipline, may I now proceed to consider first in broad outline the changes occurring in clinical investigation.

I. *Change in clinical investigation effected by increased financing.* The external appearance of clinical investigation has in recent years undergone a metamorphosis in experimental paraphernalia and the number of its devotees. This has been due largely to financing, a reflection not only of our affluence as a nation, but also of the increased support from a public awakened to the accomplishments of medical research. During the last twenty years, total appropriations for medical research have increased from 40 to over 700 million dollars per year, and federal support has increased from 7 per cent in 1940 to over 50 per cent of the total appropriations at present. During the same interval the number of physicians employed as teachers and investigators is estimated to have increased by one and one-half times (1). These figures indicate that the resources available to the individual investigator have increased approximately tenfold. It has meant what we are all well aware of: a growth of research units bristling with technical personnel and equipment.

Under this sponsorship we see the emergence of the professional investigator, the man who has set out to make a career of research and who is eminently qualified to take advantage of the facilities at his disposal. By contrast, the more amateurish scientist, unprepared for this transition, has often floundered, exhausting his energies on complicated instruments which he has been unable to control. It is equally important, however, that this new brand of trained investigator should have an understanding of clinical medicine if he is to concern himself with human disease; else his so-called clinical research may become perverted to elegant but irrelevant laboratory studies. The difficulty of riding these two horses of clinical and laboratory know-how is surpassed only by the opportunities offered. In the programs of these three societies there is ample testimony of the effective application of basic science to clinical problems which has become possible through modern facilities and subsidized training. The speed of modern communication and the resources available to him also permit the investigator to seek out unusual clinical opportunities, regardless of their geographic location. This span of research activity is well illustrated by the recent studies of abnormalities in hemoglobin synthesis which extend from the chemical dissection of the amino acid sequence of the molecule through clinical manifestations and on to population genetics. There is truly a spacious arena for research before us. In the past the clinical investigator had more severe limits and therefore often applied himself to a diversity of clinical problems which increased the breadth of his medical knowledge. Today project-jumping is wasteful and greater contributions are generally made by examining the many dimensions of a single subject. This change in shape from the broad and limited approach to the study in depth is a natural one for the investigator. With all of this have come as well certain obligations:

II. *New responsibilities incurred by the scientist.* While it is fine to discuss what money has done, one should be mindful that the success of this high finance in research depends to a considerable degree on its administration. The National Institutes of Health have been placed in a position of responsibility in this connection. It is appropriate that we acknowledge the skill and wisdom with which the administrative staff of the Public Health Service has managed this funding program. It has been accomplished without infringing on the integrity of academic institutions or the independence of the individual investigator. In the general program one might underline the emphasis which has been placed on support of the man through career investigatorships and long-term general support, and on support of clinical facilities in the form of special research wards, where

studies on man may be carried out under optimal conditions. These measures have provided solidity to the new form of clinical investigation. The system of reviewing research and training projects relies heavily on advice by scientific consultants and has operated in such a manner as to win the confidence of the scientific community. Its merit, I believe, lies in the number of competent scientists who are continually rotating on its panels. I am unconvinced that one acquires better vision in the selection of productive and creative work through prolonged exposure to the task of grant selection; rather, experience hardens the artificial criteria by which requests are excluded. At any rate, the procedure by which deserving grant requests are matched with available funds is a democratic process in which I believe we should accept and indeed cherish our responsibility to participate.

Federal support of research also brings the public at large into closer relationship to the scientist. This relationship requires, on the part of the scientist, a conscience sensitive to the health needs of the people, and on the other hand, an understanding by the people of the nature of basic and applied research. The distortion of communication of scientific information to the public is a matter of serious consequence, for it may lead to faulty opinions by the public as to how and where research should be pursued. Such faulty opinions may gain expression through the undue direction of appropriations and an attempt to deploy scientists to special objectives. Problems arise through lack of understanding rather than through any difference in ultimate objectives of scientist and public. There is need for better understanding of the limitations and wastefulness of crash programs and the importance of cultivating the new idea which cannot be planned. We cannot escape the necessity of improving the transmittance of both the facts and philosophy of research to the public if clinical investigation is to proceed along orderly lines.

III. *Effects on the Society.* I will conclude with a few remarks on the relation of the American Society for Clinical Investigation to this remodeling process. The product of consequence of our Society lies in the bibliographies of our members and we should express satisfaction in the activities of our journal in which these studies ultimately find their place. The annual meeting constitutes a second medium of exchange of information. The pattern of our meetings undergoes continued modification to meet the expansion of research. This year in addition to the general session there were seven conjoint specialty sessions held with the American Federation for Clinical Research; and the total number of papers presented from our Society this year is 59, or 28 per cent of the abstracts submitted. I do not believe that suitable attention has been paid in presentation of these papers to the difference in audience between the general and specialty sessions, and therefore have invited the essayists today to make their papers understandable to the general audience. While it has been held by most members that the formal activities of our Society be limited to the annual meeting and journal, it seems most appropriate that various groups be organized in association with these meetings which cross society lines in dealing with more specific matters of research importance.

The major concern of our Society in these years is the question of membership, as presented so clearly last year by Dr. Berliner. The changing shape of clinical investigation has forced two problems on us. The first is proper selection of candidates. As clinical research expands to encompass the facts and concepts of contiguous scientific disciplines, it becomes increasingly hard to compare individuals and to define specific merits in the clinical investigator. With Berliner, I would hope for the development of an effective mechanism for scientific evaluation of candidates which would ease the heavy burden on your council. The second problem is that of numbers. The Society takes pride in the great distinction of its past members and strives for a similar standard of excellence in the future. It has been argued that to increase the number of new members elected each year would run the danger of lowering standards. The natural extension of this argument is a limited honorary society which ignores the growth of clinical investigation. Such a small society cannot easily embrace new currents of research and blocks unique individuals and those in overlapping areas of science from membership. Also, if one is forced to choose people who have already arrived, one may end up with a dignified society which is going nowhere. I realize that the last statement is a provoking one, but I cannot believe that this Society has adequately met the challenge of expansion and change in contemporary investigation and would personally urge an orderly adoption of appropriate increases in membership. The proposed change in constitution to be voted on today would provide a step in that direction.

REFERENCE

1. Federal Support of Medical Research. Report of the Committee of Consultants on Medical Research to the Subcommittee on Departments of Labor and Health, Education, and Welfare of the Committee on Appropriations, United States Senate, Eighty-sixth Congress, Second Session. May, 1960.

PAPERS PRESENTED AT THE FIFTY-THIRD ANNUAL MEETING 1961

1. On the Site of Viral Interference. MONTO HO, Pittsburgh, Pa. (introduced by Maxwell Finland). (1049)
2. The Relative Abundance of Viral Receptors: An Explanation of the Differential Susceptibility of Suckling and Adult Mice to Coxsackie B_1 Infection. CALVIN M. KUNIN and NORMA E. HALMAGYI, Charlottesville, Va. (introduced by William S. Jordan, Jr.). (1055)
3. Pyrimidine Biosynthesis in Man: Suppression *In Vivo* by Anti-neoplastic Agents. MITCHELL RABKIN, ELIZABETH FREDERICK, MYRON LOTZ and LLOYD H. SMITH, JR.,* Boston, Mass. (1073)
4. Leukemic Cell Proliferation as Determined by *In Vitro* Deoxyribonucleic Acid (DNA) Synthesis. CHARLES G. CRADDOCK * and GEORGE S. NAKAI, Los Angeles, Calif. (1031)
5. Abnormalities of Erythrocyte and Plasma Lipids in Acanthocytosis. PETER WAYS, CLAUDE F. REED and DONALD J. HANAHAN, Seattle, Wash. and Rochester, N. Y. (introduced by Cyrus E. Rubin). (1088)
6. Active Transport of Iron *In Vitro* by Duodenal Segments and the "Mucosal Block." JAMES G. MANIS and DAVID SCHACHTER,* New York, N. Y. (1060)
7. Response to Repeated Platelet Transfusions from the Same Donor. EMIL J. FREIREICH, ALLAN KLIMAN, LAWRENCE A. GAYDOS and LESLIE R. SCHROEDER, Bethesda, Md. (introduced by C. Gordon Zubrod). (1039)
8. Bile Acid Fever and Inflammation in Man. ROBERT H. PALMER, PAUL B. GLICKMAN and ATTALLAH KAPPAS, Chicago, Ill. (introduced by Richard L. Landau). (1069)
9. Myocardial Blood Flow as Indicated by the Disappearance of NaI^{131} from the Heart Muscle. WILLIAM HOLLANDER, IRVING M. MADOFF and ARAM V. CHOBANIAN, Boston, Mass. (introduced by Robert W. Wilkins). (1050)
10. Genetic Control of the Ability of Human Blood to Neutralize Angiotensin and Its Relationship to Essential Hypertension. J. EDWIN WOOD,* Augusta, Ga. (1090)
11. The Influence of Arterial Pressure on the Antihypertensive Action of a Normal Kidney, a Biological Servomechanism. LOUIS TOBIAN,* BEVERLY WINN and JEANETTE JANECEK, Minneapolis, Minn. (1085)
12. Relationship of Bacteriuria to Hypertension: An Epidemiological Study. EDWARD H. KASS,* WILLIAM E. MIALL and KENNETH L. STUART, Boston, Mass., Penarth, Wales and Jamaica, W. I. (1053)
13. The Alveolar Diffusing Volume and the Water Space of the Pericapillary Tissues of the Lungs as Measured by Multiple Indicator Dilution Curves. LLOYD RAMSEY, W. PUCKETT, A. JOSE and W. LACY, Nashville, Tenn. (introduced by C. R. Park). (1074)
14. Dominant Role of Pulmonary Vascular Engorgement as a Determinant of Dyspnea. H. PAGE MAUCK, JR. and WILLIAM SHAPIRO, Richmond, Va. (introduced by John L. Patterson). (1061)
15. The Determination of Intracellular pH in Normal Human Subjects. EUGENE D. ROBIN,* PHILIP A. BROMBERG and ROBERT J. WILSON, Pittsburgh, Pa. (1076)
16. Evidence for a Renal Tubular Amino Acid Transport System Common to Glycine, L-Proline and Hydroxy-L-proline. CHARLES R. SCRIVER, IRWIN A. SCHAFER and MARY L. EFRON, Montreal, Canada and Boston, Mass. (introduced by John C. Beck). (1080)
17. The Effect of Blood Glucose on Circulating Bound and Free Insulin. HARRY N. ANTONIADES, KARE GUNDERSEN and HUGH PYLE, Boston, Mass. (introduced by Charles A. Janeway). (1022)
18. Relationship of Sulfhydryl Inhibitors and Reductive Conditions to the Action of Insulin on Adipose Tissue. W. S. LYNN, JR.,* EUGENIA EARNHARDT and ROSE BROWN, Durham, N. C. (1059)
19. Effects of P_{CO_2} and pH on the Plasma Binding, Turnover, and Tissue Uptake of Thyroid Hormones. MILTON W. HAMOLSKY * and MYRON STEIN, Boston, Mass. (1047)
20. Alterations in Steroid Hormone Secretion Produced by Inhibition of Cholesterol Biosynthesis. JAMES C. MELBY, MARLYN ST. CYR and S. L. DALE, Little Rock, Ark. (introduced by Richard V. Ebert). (1063)
21. Mechanism of the Anabolic Effect of Testosterone. J. D. WILSON, Dallas, Tex. (introduced by Marvin D. Siperstein). (1088)
22. Antidiuretic Hormone-like Effects of Adenosine-3′,5′-phosphate (Cyclic AMP) and Theophylline in the Toad Bladder. JACK ORLOFF * and JOSEPH HANDLER, Bethesda, Md. (1068)
23. Human Serum Growth Hormone: Measurements of Concentration and Turnover with a Radioimmunoassay. ROBERT D. UTIGER, MARY L. PARKER and WILLIAM H. DAUGHADAY,* St. Louis, Mo. (1086)
24. The Mechanism of Aldosterone Stimulation Following Hemorrhage in the Hypophysectomized Dog. PATRICK J. MULROW and WILLIAM F. GANONG, New Haven, Conn. and San Francisco, Calif. (introduced by J. W. Hollingsworth). (1065)
25. Biochemical Detection and Histological Study of Muscular Dystrophy in the Preclinical Stage. CARL M. PEARSON, SUDHIR R. CHOWDHURY and WILLIAM M. FOWLER, Los Angeles, Calif. (introduced by William N. Valentine). (1070)
26. The Effects of Guanethidine on Triiodothyronine-induced Hyperthyroidism in Man. THOMAS E. GAFFNEY and EUGENE BRAUNWALD, Bethesda, Md. (introduced by Robert W. Berliner). (1040)

* Member.

( ) Page number of abstract.

ABSTRACTS

Effect of Weight Gain During Adult Life on Serum Triglyceride Concentrations of Normal Man. MARGARET J. ALBRINK,* J. WISTER MEIGS AND MORRIS GRANOFF, New Haven, Conn.

The finding of high serum triglycerides in patients with coronary artery disease prompted a study of the association between serum lipids and various traits of 215 apparently healthy male factory employees between 30 and 70 years of age. Significant elevations of serum triglycerides were associated with advancing age, obesity, family history of diabetes or coronary artery disease, and weight gain during adult life. The mean serum triglyceride concentration of 78 men who had gained less than 10 pounds since age 25 was 4.6 mEq per L compared to 7.1 mEq per L for the 137 men who had gained more than 10 pounds, a highly significant difference. The weight-stable group did not show an increase in later decades as did the weight gainers. Although stocky men of stable weight had somewhat higher triglycerides than lean men, those who achieved a moderate degree of corpulence [estimated as height/(weight)$^{-3}$ index] through weight gain during adult life had a mean triglyceride concentration nearly twice that of men who reached young manhood with this degree of adiposity but gained no further weight during adult life. The tendency for low triglycerides in men with negative family histories noted for the entire group was not found in the weight-gainers. Among the weight-stable men, however, those with a negative family history had significantly lower triglycerides than those with a positive family history and as a group had the lowest triglycerides except for the leanest 7 per cent in whom not a single instance of triglyceride concentration above 5.5 mEq per L was encountered regardless of family history. Serum cholesterol varied roughly with triglycerides but in general the differences between the various groups was of a lower degree of significance. It was concluded that weight gain during adult life was the single most important factor associated with hyperglyceridemia in normal men.

Metabolic and Circulatory Effects of Epinephrine in the Forearm of Man. REUBIN ANDRES,* MARCEL BALTZAN, GORDON CADER AND KENNETH L. ZIERLER,* Baltimore, Md.

Consensus of recent literature is that infusion of epinephrine i.v. in man causes sustained increase in forearm blood flow and in systemic blood lactate concentration, but intra-arterial infusion causes only transient increase in forearm blood flow and no increase in lactate production by peripheral tissues. It has therefore been postulated that epinephrine itself lacks sustained vasoactive effect and even glycogenolytic potency in man and that some active intermediate is formed by it to explain its i.v. effects. Furthermore, the effect of epinephrine on net postassium movement in muscle is in dispute, and other studies in a variety of preparations suggest that epinephrine inhibits glucose uptake by skeletal muscle. We have tried to resolve these reports, which are either internally inconsistent or at variance with reports of epinephrine effects in laboratory animals or in isolated systems, by carefully controlled observations in 17 subjects in whom epinephrine was administered into the brachial artery (0.002 μg per kg body wt per minute for 25 minutes) to achieve an arterial concentration of about 4 μg per L. This dose exerted no systemic effect (constant arterial glucose, lactate, K and free fatty acids) but it produced: 1) sustained doubling of forearm blood flow (so that no vasoactive intermediate need be postulated), 2) 10-fold increase in forearm lactate production (so that it is glycogenolytic in man), 3) small increase of no statistical significance in glucose uptake, 4) large K uptake, and 5) the anticipated increase in FFA release by forearm adipose tissue.

The Effect of Blood Glucose on Circulating Bound and Free Insulin. HARRY N. ANTONIADES, KARE GUNDERSEN AND HUGH PYLE, Boston, Mass. (introduced by Charles A. Janeway).

Earlier reports have presented evidence that insulin circulates in the peripheral blood in two forms—a biologically active "free" insulin and a biologically inactive "bound" form. The bound insulin is complexed to basic proteins and can be dissociated *in vitro* into biologically active free insulin by centrifugation at pH 10 or by incubation with adipose tissue extracts. Examination of the ratio of free and bound insulin in nondiabetic subjects shows that this ratio does not depend upon a pure physicochemical equilibrium. Present studies suggest that the ratio is related to the blood glucose level. Examination of free and bound insulin before and after glucose administration shows that a rise in blood glucose results in a dissociation of the bound insulin. Following the intravenous administration of glucose, there is a rapid dissociation of bound insulin resulting in an increase of free insulin. The free insulin falls with the decreasing blood glucose level. At the end of 4 to 5 hours, the ratio of "bound" insulin to "free" again returns to preglucose administration levels. In diabetic patients, however, the rate of dissociation of bound insulin, following glucose administration, is significantly slower and does not reach the nearly complete dissociation found in nondiabetic subjects.

* Member.

The Effect of 17-Ethyl-19-nortestosterone and Icterogenin on the Hepatic Metabolism of Bilirubin in Normal and Gunn Rats. IRWIN M. ARIAS, SIDNEY GOLDFISHER, ALEX B. NOVIKOFF AND EDWARD ESSNER, New York, N. Y. (introduced by David J. Hamerman).

Conjugated hyperbilirubinemia, bilirubinuria and relatively normal hepatic histology have been regularly observed in humans after 17-ethyl-19-nortestosterone (Nilevar) administration and in sheep and rabbits after treatment with Icterogenin, an alkaloid obtained from *Lippia rehmanni*. The administration of Nilevar or Icterogenin to Wistar rats resulted in significant reduction in the maximal rate of excretion of bilirubin in the cannulated bile duct following a constant intravenous infusion of unconjugated bilirubin. The maximal rate of excretion of bilirubin in the cannulated bile duct was similarly reduced in Wistar and homozygous Gunn rats following constant intravenous infusions of conjugated bilirubin. Hepatic uridine diphosphoglucose dehydrogenase and glucuronyl transferase activities were unaffected by treatment with either Nilevar or Icterogenin. Although the livers of treated animals appeared normal in routine histological sections, marked changes were apparent in both cytochemical and electron microscopic preparations. The bile canaliculi (excretory surfaces) showed dilatation and fragmentation accompanied by decreased ATPase activity, increased alkaline phosphatase activity and considerable loss of microvilli. Changes also occurred elsewhere in the cell membrane and in acid phosphatase-rich lysosomes. These observations suggest that Nilevar and Icterogenin interfere with the mechanism by which conjugated bilirubin is transported from the liver cell into the bile.

Alterations in Granulocyte Kinetics Induced by Epinephrine, Exercise, Endotoxin and Prednisone. JOHN W. ATHENS,* SPENCER O. RAAB, OTTO P. HAAB, HELEN ASHENBRUCKER AND GEORGE E. CARTWRIGHT,* Salt Lake City, Utah.

The influence of epinephrine, exercise, endotoxin and prednisone on the total blood granulocyte pool (TBGP), circulating granulocyte pool (CGP), marginal granulocyte pool (MGP), half-time disappearance of granulocytes from the TBGP ($T_{\frac{1}{2}}$), and the granulocyte turnover rate (GTR) has been studied in normal subjects. Granulocytes were labeled *in vitro* by the method described previously. Epinephrine infusion (5 subjects) or acute exercise (4 subjects) caused a shift of granulocytes from the MGP to the CGP with no change in TBGP, $T_{\frac{1}{2}}$ or GTR. In 2 of 6 subjects given bacterial endotoxin a neutropenia occurred 1.5 hours after endotoxin injection, but the TBGP was unchanged. The neutropenia thus reflected a shift of granulocytes from the CGP to the MGP. A neutropenic phase was not detected in the remaining 4 subjects. Five hours after endotoxin injection all 6 subjects had a leukocytosis and the TBGP increased twofold. Endotoxin injection did not alter the granulocyte radioactivity curve during the neutropenic period, but coincident with the rise in granulocyte count a precipitous decrease in radioactivity occurred. These findings suggest that endotoxin produced neutrophilia by causing an influx of unlabeled granulocytes from the bone marrow into the TBGP. It may also be inferred that in subjects who develop leukopenia there is a transient shift of granulocytes from the CGP to the MGP. Five subjects were given prednisone for 11 days. The TBGP increased 67 per cent and the $T_{\frac{1}{2}}$ was prolonged. The GTR was unchanged. Rapid infusion of hydrocortisol produced a marked neutrophilia but there was no sharp decrease in the radioactivity curve. These data suggest that steroid neutrophilia does not result from an influx of granulocytes from the marrow.

Leukocyte D-Antigenicity Studied by Means of a Specific I^{131}-labeled Anti-D Antibody. ARTHUR E. BARNES, HERNANDO SARASTI AND WALLACE N. JENSEN,* Pittsburgh, Pa.

Specific I^{131} anti-D antibody eluates (I*anti-D) were prepared from the globulin fraction of high-titered anti-D serum. The I*anti-D was isolated by adsorption to D-positive (ccDee) erythrocytes and by subsequent elution with citric acid buffer. Immunologic specificity of the I*anti-D was demonstrated by 70 to 80 per cent total uptake by D-positive erythrocytes as compared with 2 to 10 per cent nonspecific adherence to D-negative erythrocytes and test tube. The amount of erythrocyte D-antigen necessary for a significant I*anti-D uptake above nonspecific adherence varied and was estimated by the disappearance of differences between the I*anti-D uptake on equal numbers of D-negative and D-positive erythrocytes in serial dilution. A 3 per cent greater I*anti-D uptake by D-positive erythrocytes than by D-negative erythrocytes was the minimal difference considered significant. Leukocyte suspensions isolated by differential sedimentation from 7 normal and 2 leukemic D-positive donors showed no I*anti-D uptake not attributable to the I*anti-D bound by contaminant erythrocytes or by nonspecific adherence. The amount of I*anti-D bound by the contaminant erythrocytes and nonspecific adherence was estimated by the amount of I*anti-D bound by the same number of erythrocytes as measured in each leukocyte suspension. Leukocytes from 5 other normal and 1 other leukemic donor showed small I*anti-D uptakes not attributable to D-positive erythrocyte contamination or nonspecific adherence; however, the mean I*anti-D uptake of this group was only 3.6 per cent of the uptake by equal numbers of D-positive erythrocytes. In addition, paired leukocyte suspensions from three D-positive and three D-negative donors had similar I*anti-D uptakes. Furthermore, serial dilutions of these leukocyte suspensions failed to show the attenuation of the total I*anti-D uptake expected when specific D-antigen sites were decreased in number. These investigations strongly suggest that leukocytes have very few or possibly no specific D-antigen sites.

Metabolic Functions of Vitamin B_{12}: The Possible Relationship of Megaloblast Formation to Unbalanced Growth in Bacteria Requiring Vitamin B_{12}. WILLIAM S. BECK,* Boston, Mass.

Although the megaloblast is a familiar morphological entity, few precise statements can be made about the mechanism of megaloblast formation. This shortcoming is attributable to 1) technical difficulties in obtaining homogeneous megaloblast populations; 2) faulty conclusions from existing data; and 3) incomplete knowledge of the nutritional basis of megaloblast formation in different species, of the means by which nutritional repletion reverses the process, of the biochemical functions of vitamin B_{12} and pteroylglutamic acid, and of the nutritional interactions of bone marrow elements. Our studies of the role of vitamin B_{12} in bacterial nucleic acid metabolism have implicated B_{12} in the biosynthesis of deoxyribonucleosides or deoxyribonucleotides. It appears that B_{12} participates (directly or indirectly?) in the reduction of ribosyl to deoxyribosyl groups, a reaction resembling other B_{12}-linked reductions (e.g., methyl synthesis) but differing from the B_{12}-linked isomerases (methylmalonyl CoA and glutamate) in which there is no net change in oxidation level and B_{12} participates directly as a coenzyme. Evidence implicating B_{12} in this conversion includes the nutritional sparing of vitamin B_{12} by deoxyribosides in lactobacilli and the observed effect of B_{12} on the production of acid-soluble deoxyribonucleosides or deoxyribonucleotides and DNA. Indications were also obtained that vitamin B_{12} has no direct role in thymine synthesis. In addition, the unbalanced growth phenomenon was observed in *Lactobacillus leichmannii* cultivated in limiting vitamin B_{12} or deoxyriboside. This is characterized by long filamentous forms in which cell division and DNA synthesis are defective while RNA and protein synthesis proceed normally. Similar results were obtained with B_{12}-starved *Euglena gracilis*. It will be shown that the biochemical characteristics of these filaments correspond with those described in the megaloblasts of pernicious anemia. They may therefore serve as useful models of the megaloblastic growth of B_{12} deficiency which is thus visualized as unbalanced growth due to diminished DNA synthesis resulting from impaired deoxyribosyl synthesis. The implications of this hypothesis will be examined.

Histochemical Topography of Anterior Pituitary: Correlations Between Enzyme Activities and Endocrine Function of Individual Cells. S. BLEICHER, M. KARNOVSKY AND N. FREINKEL,* Boston, Mass.

The responsiveness of adenohypophyseal carbohydate metabolism to insulin (Goodner and Freinkel) prompted further assessment of correlations between endocrine function and intermediate metabolism in anterior pituitary. Cryostat sections of male rat pituitary were incubated with Nitro-BT for histochemical localization of *a*) DPN- and TPN-diaphorases (DPND, TPND) as indices of electron transfer, and *b*) glyceraldehyde-3-P dehydrogenase (G3PD) and glucose-6-P dehydrogenase (G6PD) as representative enzymes of glucose disposition by glycolysis or direct oxidation, respectively. In adenohypophyses from normal animals, all cells displayed distinct TPND activity; less uniform reactivity obtained for DPND and G3PD; G6PD staining was patchy and variable. The reactions were most pronounced in the ovoid cells around the portal vessels which have been previously implicated in the secretion of gonadotropin. Their gonadotropic character was corroborated by orchiectomy. Within 3 to 4 days following castration, histochemical reactions were intensified in these cells (TPND > G6PD > G3PD > DPND) although other pituitary components were unaltered. Eventually, coincident with diffuse increase in "gonadotropes" (as judged by conventional histology), cells with augmented histochemical activity also became more numerous. Maximal reactions were seen in the signet-ring "castration cells" which appear several weeks after orchiectomy. The data indicate that *a*) pituitary cells may be differentiated by histochemical enzyme reactions; *b*) focal enzymatic changes may be associated with specific hormogenic challenges; and *c*) such localized metabolic realignments may be among the earliest indices of altered function. With regard to *c*, the heightened enzymatic activity of normal "gonadotropes" may be involved in the disparate vulnerability of gonadotropic function to diverse metabolic insults.

The Effect of Serum Albumin Infusion upon Lipid Mobilization in the Nephrotic Syndrome in Man. M. D. BOGDONOFF, J. LINHART, R. F. KLEIN AND E. H. ESTES, JR., Durham, N. C. (introduced by W. M. Nicholson).

In order to test the thesis that the interrelationship of serum albumin and the serum lipids is of specific import to the plasma lipid abnormality of the nephrotic syndrome, the following studies of lipid mobilization in man were conducted. Lipid mobilization was effected by a standardized (15 minutes; total dose, 150 μg) infusion of norepinephrine, and the pattern of change of the plasma free fatty acid (FFA) was followed. All subjects were observed in the resting, fasting state. The peak rise in FFA level is used as an index to the magnitude of lipid mobilization. Pulse, blood pressure, hematocrit, serum cholesterol, triglyceride, protein, albumin and glucose were also measured. In 20 metabolically normal individuals the mean peak FFA rise was +600 μEq per L; in 6 patients with the nephrotic syndrome the peak FFA rise was +54 μEq per L ($p < 0.01$). Blood pressure and pulse changes were similar. Following the infusion of 50 g of salt-poor human serum albumin (over a 30 minute period), the peak rise in normal subjects was further heightened to +1,000 μEq per L; in the nephrotic patients peak values were increased to +300 μEq per L. (Difference between the means, $p < 0.01$.) In two of the nephrotics serum triglycerides decreased following albumin and norepinephrine beyond the decrement accounted for by the expansion of plasma volume. In two other individuals with non-

nephrotic hypoalbuminemia, peak FFA rises were also low (+40, +50 μEq per L), and were similarly enhanced following the infusion of albumin (+250, +300 μEq per L). These studies are interpreted to indicate that the process of lipid mobilization is significantly modified by the nephrotic syndrome, and that this decreased responsiveness may be, in part, temporarily reversed by the infusion of serum albumin. It is further suggested that the hypertriglyceridemia may reflect a response to the loss of plasma FFA binding sites.

Congenital Adrenal Hyperplasia due to Deficiency of 3β-Hydroxy Steroid Dehydrogenase. ALFRED M. BONGIOVANNI* AND WALTER R. EBERLEIN,* Philadelphia, Pa.

Three unusual infants with the salt-losing form of congenital adrenal hyperplasia have been studied (one a male and two female pseudohermaphrodites). All died within the first few months of life in spite of presumably adequate therapy. Two of the infants had siblings who died with similar symptoms. Postmortem examination was performed on one of the studied cases and on the sibling of another. The excretion of total neutral 17-ketosteroids and Allen chromogens in the urine of all three cases was elevated and fell during treatment with cortisol. Pregnane-3α,17α,20α-triol, pregnane-3α,17α-diol-20-one, and pregnane-3α,17α,20α-triol-11-one, generally found in large amounts in the urine in other subjects with this form of disease, were virtually absent. Urinary Porter-Silber chromogen excretion was extremely low and no metabolites of cortisol were detected by paper chromatography of suitably prepared extracts of urine. The major urinary steroid metabolites were present in the nonglucuronoside, digitonin-precipitable fraction, recovered by solvent extraction after solvolysis in tetrahydrofuran with 10^{-2} N perchloric acid or prolonged heat hydrolysis at neutral pH and reconstitution in acidic ethanol. The predominant steroid isolated was identified as Δ^5-pregnene-3β,17α,20α-triol. Smaller amounts of Δ^5-pregnene-3β,20α-diol were also detected. Dehydroepiandrosterone accounted for approximately 25 per cent of the total 17-ketosteroids. Other ketonic steroid metabolites, tentatively identified, were Δ^5-pregnene-3β,17α-diol-20-one and Δ^5-androstene-3β,16α-diol-17-one. Certain of these metabolites have previously been isolated from the urine of subjects with adrenocortical neoplasms, but this is the first reported instance of their excretion in subjects with congenital adrenal hyperplasia. These findings indicate a third specific defect in adrenal steroidogenesis in the latter syndrome, a primary deficiency of the adrenocortical enzyme, 3β-hydroxy dehydrogenase.

Procedure for Isolation and Determination of Human Blood Angiotensin. R. BOUCHER, P. BIRON AND J. GENEST, Montreal, Canada (introduced by Vincent P. Dole).

This procedure, modifying in several aspects Paladini's method, involves: 1) direct ethanol precipitation of 100 to 400 ml of arterial or venous blood, 2) ether extraction of the filtrate, 3) successive purification of the aqueous phase by column and paper chromatography. Angiotensin, located by its Rf value, is assayed in a rat preparation for pressor activity. This procedure is sensitive to 0.005 μg. Recovery of angiotensin is quantitative. Specificity is based on the following similarities with the standard valine-5 angiotensin II asparaginyl: 1) identical Rf values in two different paper chromatographic systems, 2) identical migration rate in one paper electrophoretic system, 3) identical pressor response curve in the rat assay, 4) angiotensin added to blood or isolated from blood during infusion of this substance, shows the same characteristics as mentioned in 1, 2 and 3; 5) the angiotensin isolated from human serum after incubation with a renin preparation from renal tissue of a patient with unilateral renal hypertension, shows the same characteristics as the material isolated from hypertensive patients; 6) inactivation by trypsin. Arterial angiotensinemia during angiotensin infusion given at hypertensive rates (30 mm Hg diastolic pressure above control levels) varies between 0.035 and 0.200 μg% of blood. So far, 20 normal subjects and 40 patients with various types of hypertension have been studied. Based on the above criteria, angiotensin is present in some hypertensive patients.

An Abnormal Serum Cholesterol Ester Fatty Acid Pattern in Liver Disease. C. Y. BOWERS, JAMES E. MULDREY, JAMES G. HAMILTON AND O. NEAL MILLER, New Orleans, La. (introduced by Grace A. Goldsmith).

The ratio of free cholesterol to its esters in human serum has been used as a test of liver function. To extend these observations the cholesterol ester fatty acid pattern was determined in sera of patients with various liver diseases. Serum lipids are chromatographed on silica gel-coated glass fiber paper. Chromatography in iso-octane (20 minutes) completely separates cholesterol esters into five fractions: S, saturates, O, oleate and palmitoleate; L, linoleate; A, arachidonate and linolenate; and P, higher polyenoates. Esters are visualized as charred spots after treating with sulfuric acid followed by heating and were determined quantitatively by densitometry of the charred spots. Representative values for S, O, L and A are 25, 70, 120 and 25 mg% in normal persons and 20, 75, 45 and 20 mg% in patients with cirrhosis of the liver. This reversal of the O/L ratio has been conspicuously absent in patients with endocrine abnormalities and various lipid disorders, including patients with hypercholesterolemia on varied dietary regimens. It is probable that studies of cholesterol esters in liver disease may elucidate some of the regulating factors which influence the metabolic turnover of the fatty acid moieties.

Heterogeneity of Serum Alkaline Phosphatases: The Influence of Race, Pregnancy, Growth and Disease. SAMUEL H. BOYER, RICHARD SCHULTZ AND ROBERT WEILBACHER, Baltimore, Md. (introduced by Victor A. McKusick).

A variety of investigations have established the presence of several phosphatases in human serum. We have demonstrated 13 serum alkaline phosphatases by starch gel electrophoresis and subsequent enzyme identification by histochemical methods. Less effective resolution was obtained by cellulose column chromatography. Ten components are α_2-globulins and three are β-globulins. No single individual exhibits all components. Patterns observed in normal white adults are generally quite similar and consist of two principal zones of activity. Approximately 4 per cent of American Negroes have a group of phosphatases not observed in whites. One of these latter components possesses a mobility identical to the principal alkaline phosphatase purified from human intestine. The alkaline phosphatases associated with a variety of diseases do not usually differ electrophoretically from the normal pattern, and when different are not specific for any particular disorder. From birth to adolescence the two principal zones of phosphatase activity differ slightly from the adult pattern. Patterns appearing in children with hypophosphatasia or rickets are electrophoretically indistinguishable from the normal childhood pattern. During pregnancy additional electrophoretic zones of phosphatase activity start to appear beginning at about Week 15 of gestation. By Week 31 nearly all women exhibit the additional components, which disappear shortly after delivery. One particular component, unique to pregnancy, appeared in 36 of 57 white women who were in Week 31 of their confinement. In a similar group of 59 American Negroes this component was present in only 14 individuals. The racially associated heterogeneity of certain serum alkaline phosphatases suggests a genetically determined polymorphism. The tissue source of the various enzymes can possibly be established by comparison with purified phosphatases from several tissues and by immunochemical methods.

Stimulatory Effect of Chlorobutanol on Uridine Diphosphate Glucose Dehydrogenase. GEORGE A. BRAY AND A. H. CONNEY, Bethesda, Md. and Rochester, N. Y. (introduced by Lawrence E. Young).

Patients with essential pentosuria have a metabolic block in the glucuronic acid pathway leading to the excretion of large amounts of L-xylulose. Aminopyrine stimulates the excretion of L-xylulose in these patients presumably by stimulating this pathway. Administration of aminopyrine, chlorobutanol, barbiturates and several other drugs to rats has the dual property of accelerating the formation of ascorbic acid through the glucuronic acid pathway and of increasing the activity of certain drug-metabolizing enzymes in liver microsomes. *In vitro* studies with rat liver preparations have shown that the effect of chlorobutanol administration *in vivo* cannot be explained by altered activities of hepatic glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, uridine diphosphate glucose (UDPG) pyrophosphorylase or by altered activities of the enzyme systems that convert D-glucuronolactone to L-gulonolactone or which convert L-gulonolactone to L-ascorbic acid. However, chlorobutanol administration increases the activity of UDPG dehydrogenase by about 100 per cent. Chlorobutanol administration also has a marked effect in stabilizing UDPG dehydrogenase. This enzyme, when obtained from control rats, is unstable if allowed to age at 4° C for 5 days but is stable when obtained from chlorobutanol-treated rats. The enzyme preparations obtained from control rats can be stabilized by the addition of UDPG or uridine diphosphate glucuronic acid (UDPGA) but not by the addition of ATP or chlorobutanol. It is possible that increased activity of UDPG dehyrogenase may make more UDPGA available for ascorbic acid synthesis and for drug glucuronide formation. Administration of chlorobutanol and other drugs that stimulate ascorbic acid synthesis via the glucuronic acid pathway also stimulates the activity of enzyme systems in liver microsomes that metabolize drugs. The mechanism by which drug administration elicits these adaptive responses is being investigated.

Biosynthesis of Urea in Kidney Tissue Without Exogenous Substrate. WILLIAM A. BRODSKY* AND H. C. HUANG, Louisville, Ky.

Urea and ammonia concentrations of kidney cortices of dogs were observed under *in vitro* conditions. Homogenates or slices of cortex were immersed in K-rich Ringer's solutions with and without added substrates. Urea concentration increased from initial levels of ca. 15 to 19 μmoles per g in 1 to 2 hours. Free ammonia, measured simultaneously in the same aliquots increased from 3 to 8 μmoles per g. Rate of formation of urea in slices was increased slightly by addition of citrulline, glutamate, Mg^{++}, and cytochrome C. In one case, urea concentration remained fixed when the tissue was incubated in Na-rich Ringer's. In another, urea concentrations remained fixed when tissue was incubated in a citrulline glutamic acid Ringer's of pH 4. Findings confirm and extend those of Krebs, Borsook and Cohen who found urea or arginine formation in kidney slices and homogenates, but only with added substrate. This might explain changes of urea clearance in sheep on high and low protein diets (Schmidt-Nielsen), as well as the high urea/inulin ratios in micropuncture samples of distal convoluted tubules (Lassiter, Gottschalk).

Chromatographic Studies of Endogenous Pyrogen. NORMAN K. BROWN AND ROBERT G. PETERSDORF, Seattle, Wash. (introduced by William M. M. Kirby).

Endogenous pyrogen (EP) has been found in the blood stream of animals given bacterial endotoxins,

myxoviruses, tuberculin-sensitive animals challenged with old tuberculin, and in experimental pneumococcal, streptococcal, and staphylcoccal infections. EP is presumably derived from injured leukocytes, and leukocytic exudates have been found pyrogenic for animals of the homologous species. Precise biochemical characterization of EP has not been accomplished although available data indicate that it is a protein. In the present studies column chromatography was employed to purify EP, to delineate its biochemical properties and to determine any possible biochemical relationship between serum and leukocytic EP. EP was produced by intravenous administration of typhoid vaccine to rabbits. Two-hour serum was obtained and dialyzed to 0.01 M ionic strength. Dialyzed serum was placed on columns of diethylaminoethyl-cellulose (DEAE) in Tris-succinate buffer at pH 8.2 or in acetate buffer at pH 4.5 to 5.4. Stepwise elution was accomplished with increasing concentrations of NaCl. Tests for pyrogenicity in rabbits, paper electrophoresis, and biuret protein determinations were performed on each eluted fraction. EP was consistently eluted from columns at pH 8.2 with salt concentrations of 0.05 to 0.1 M. Under these conditions, only fractions containing α- and β-globulins were pyrogenic. More efficient purification was obtained with columns at pH 4.5, where EP was adsorbed in association with α-globulins. The remaining serum proteins, comprising about 80 per cent of the original sample, had no affinity for DEAE at this pH. These results suggest that serum EP resides in the electrophoretic α-globulin fraction. This method of selective absorption of EP from nonpyrogenic serum proteins may offer a valuable approach to further purification of EP and its characterization in various types of fever. Preliminary chromatographic studies of EP from leukocytic exudates have paralleled those of EP derived from serum.

A New Parameter in Evaluation of Normal and Abnormal Tissue Slice Metabolism. EDWARD A. BRUNNER AND M. A. SPIRTES, Philadelphia, Pa. (introduced by Joseph R. Di Palma).

The rate of anaerobic glycolysis of normal liver slices can be increased 5- to 10-fold by immersing the slices in hypertonic cofactor-enriched medium. This marked increase in the $Q^{N_2}_{CO_2}$ was found to be accompanied by a comparable increase in the production of lactate. Analysis of the various cofactors in the medium by exclusion experiments showed that hypertonicity, ATP and DPN were the agents responsible for the increased rate of glycolysis. By separating the slices from the surrounding medium following a 10-minute period of preincubation and then determining the glycolytic rate in the latter alone, it was demonstrated that a large part (about 50 per cent) of the increased glycolytic activity was present outside the slice if fructose 1,6-diphosphate was used as substrate. No glycolytic activity could be demonstrated in the surrounding medium when glucose was added as substrate. Thus, some leakage of glycolytic enzymes occurred into the hypertonic-enriched medium, but at least one of the first three enzymes of the glycolytic series either did not leak or was destroyed outside the slice. The possibility was next investigated that the hypertonic-enriched medium might unmask a metabolic change in an abnormal tissue slice which had not manifested itself in Ringer's solution. Liver slices from hyperthyroid animals are known to show a glycolytic rate similar to those from normal animals, in Ringer-type solutions. Utilizing the hypertonic cofactor-enriched medium it was possible to demonstrate a metabolic difference between the two types of slices, one which heretofore could not be demonstrated. The hyperthyroid slices resisted the stimulation to glycolysis which the enriched medium was able to induce in normal liver slices; they maintained a glycolytic rate similar to that which they demonstrated in the Ringer-type medium. In investigating the metabolism of tissues from animals with various physiological abnormalities, the possibility of using experiments similar to those outlined above should be considered. In this way it may be possible to show changes in other parameters where such changes have not in the past been found using a Ringer-type medium.

Changes in Erythrocyte Cation Concentration in Chronic Respiratory Insufficiency. C. E. BUCKLEY, III AND H. O. SIEKER,* Durham, N. C.

Patients with chronic respiratory insufficiency often tolerate marked hypercapnia but may develop uncompensated respiratory acidosis without striking change in the elevated arterial carbon dioxide tension. Serum electrolyte alterations in such patients are recognized, but changes in intracellular cation concentrations have not been investigated. To assess the intracellular changes in these ions, sodium and potassium concentrations were determined in erythrocytes and plasma. Observations were made on 14 normal subjects and on 62 patients with varying degrees of respiratory insufficiency. The mean erythrocyte potassium value for 14 controls was 97.4 ± 1.30 mEq per L of red blood cells. Patients with compensated respiratory acidosis (arterial pH above 7.34) had a mean erythrocyte potassium value of 107.0 ± 2.28 mEq per L, while those with uncompensated respiratory acidosis (arterial pH below 7.35) averaged 95.5 ± 2.52 mEq per L. Erythrocyte sodium concentrations in patients with severe respiratory acidosis and cor pulmonale ranged up to 74.6 mEq per L, as compared to a mean of 14.0 mEq per L for controls. Congestive heart failure and decompensated respiratory acidosis were associated with lowering of the erythrocyte potassium concentration in proportion to the severity of the heart failure. Low plasma sodium values were observed in some of the patients in this group. The total of the two cations, sodium and potassium, ranged as high as 181.5 mEq per L in this group as compared to a mean of 111.4 mEq per L for controls. These data suggest that the erythrocyte sodium and potassium concentrations portray the reten-

tion and distribution of cations secondary to bicarbonate retention in chronic respiratory insufficiency. The shift of sodium into the red cell and the associated potassium loss reflect decompensation with respiratory acidosis and congestive failure.

The Effect of Mineralocorticoids on Renal Handling of Potassium. ROBERT CADE AND ROBERT SHALHOUB, New York, N. Y. (introduced by Ralph E. Peterson).

According to Berliner, potassium excreted in urine enters the tubular fluid as a result of ion exchange in distal tubules and collecting ducts. Exchange of Na for K and H ions in the distal tubule can be increased by administration of mineralocorticoids. We have investigated the relationship between Na load and K excretion by infusing Na_2SO_4 into normal and adrenalectomized humans and dogs and into humans with spontaneously occurring adrenal insufficiency and have found that activity of distal ion exchange processes is related to dose of administered mineralocorticoid. In normal and in adrenalectomized animals with mineralocorticoid replacement, potassium excretion is directly proportional to Na load until a K excretory plateau is reached; further increases in Na load then have no effect on K excretion. The ratio UV_K/UV_{Na}, when UV_K is below the excretory plateau, is a function of mineralocorticoid levels. With large doses of steroids, ratios approaching 2 are obtained; with decreasing doses, the ratio decreases progressively to less than 0.1. Adrenalectomized animals without mineralocorticoid replacement do not vary K excretion with alterations of Na load; when the glomerular filtration rate is changed, however, excretion of K varies in direct proportion to the alteration of filtered K. In the absence of mineralocorticoid we interpret this data as indicating complete inactivity of distal ion exchange; all excreted potassium results from filtration and incomplete reabsorption. When mineralocorticoid is present, the activity of distal ion exchange is related to the steroid level. The maximal K excretory plateau is affected by body stores of K and other factors but cannot be raised by increasing steroid doses.

The Altered Distribution of the Bile Acid Pool in Obstructive Jaundice and its Correction, Including Relief of Pruritus, with a Bile Acid Sequestering Resin. JAMES B. CAREY, JR., Minneapolis, Minn. (introduced by C. J. Watson).

The hypothesis has long been held that the pruritus of jaundice is related to increased concentrations of bile acids in blood. The bile acid pool is normally confined almost entirely to the enterohepatic circulation; only small amounts escape in feces and even smaller quantities occur in systemic blood. To investigate the altered distribution and excretion of bile acids in pruritic jaundice and the effect of removing them from the enterohepatic circulation, total serum bile acid concentrations were measured spectrophotometrically and fecal deoxycholic acid was quantitated after isolation by column chromatography. Bile acids were removed from the enterohepatic circulation by feeding an anion exchange resin (MK-135) which binds them in a nonabsorbable complex. The fecal excretion of deoxylcholic acid was increased from 54 to 500 mg per day in a healthy adult fed 10 g daily. Resin administration to six jaundiced patients with severe pruritus and hyperbileacidemia (24 to 114 μg per ml) lowered serum bile acids and relieved pruritus in each patient. One, a 5 year old boy, with congenital biliary atresia and unrelenting pruritus had total serum bile acids of 115 μg per ml and excreted <1 mg deoxycholic acid per day. Resin feeding lowered serum bile acids to 20 μg per ml and increased fecal deoxycholic acid to 15 mg daily. Pruritus ceased for the first time in his life. In two patients resin withdrawal increased serum bile acids and pruritus returned; refeeding again lowered serum bile acids and relieved pruritus. These observations demonstrate that the altered distribution of the bile acid pool in incomplete biliary obstruction can be partially corrected by a resin which removes bile acids from the enterohepatic circulation, thus increasing the fecal excretion and lowering serum concentrations. The latter is associated with relief of pruritus, indicating that bile acids play an important role in the pruritus of jaundice.

Transient Excretion of Phosphate after Injection of its Potassium Salt. GASPAR CARRASQUER, Louisville, Ky. (introduced by J. Murray Kinsman).

Renal secretion of phosphate was observed in dogs following injection of KH_2PO_4 into the renal artery, using the technique of Chinard. Nembutalized dogs were given priming and maintenance infusions of mannitol, creatinine, KH_2PO_4 and NaH_2PO_4, in amounts sufficient to maintain high steady-state levels of urine flow, and to maintain constant levels of creatinine (1 to 2 μmoles per L) and of phosphate (3 to 5 μmoles per L) in plasma. After 40 to 60 minutes of maintenance infusion, instantaneous injection of KH_2PO_4 and creatinine (500 μmoles each) was delivered into one renal artery. Following injection, 20 to 30 aliquots of urine were obtained at 6 to 18 seconds each. The pattern of phosphate excretion as compared with creatinine excretion was similar to that obtained when NaH_2PO_4 was injected into the renal artery with elevated plasma phosphate. In short, the transient clearance, or increment of phosphate excreted per unit injected per one renal circulation exceeded that of creatinine during both upsweep and downsweep of the excretory response.

Disorders of Potassium Metabolism in Liver Disease. T. H. CASEY, W. H. J. SUMMERSKILL AND A. L. ORVIS, Rochester, Minn. (introduced by H. R. Butt).

Isotope studies were made in 37 patients with cirrhosis and hepatitis to define the postulated importance of disordered potassium metabolism in complications associated

with liver disease. Conflicting relationships between serum potassium (K_S), exchangeable potassium (K_E), exchangeable sodium (Na_E), total body water (TBW), body weight (BW) and dry body weight (DBW), and variable responses to administration of potassium salts, hindered identification of potassium depletion. Serial changes in K_E and K_E/DBW (30 patients) were most valid in defining metabolic interrelationships. Potassium depletion (low K_E and K_E/DBW) and the ability to retain potassium quickly (12 patients) were related to severity of the liver disease, rather than to ascites or diuresis with its treatment (16 patients). An association between ammonia and potassium metabolism was indicated in 8 noncomatose patients by increasing fasting arterial ammonia concentrations (NH_4A) in relation to decreasing K_E/DBW. In hepatic coma, changes in the EEG were related either to changes in NH_4A or to changes in K_E, where one value remained relatively constant. Extracellular pH was not a factor. During chlorothiazide therapy, clinical and EEG changes of coma were associated with elevation of NH_4A without changes in K_E. A direct effect of chlorothiazide on ammonia metabolism was also demonstrated by significant elevation of NH_4A at 60 minutes after the drug was given in ammonia tolerance tests performed with and without chlorothiazide in 11 patients. In 14 patients with disorders of water and electrolyte metabolism, phases of dilution and azotemia were demonstrated. Hypokalemia was primarily due to dilution (shown by changes in serum sodium (NA_S) related to $Na_E + K_E$/TBW. In azotemia (8 patients), hyperkalemia and ECG changes developed without potassium retention (increase in K_E) and despite greater dilution. Since falls in Na_S and rises in K_S were greater than could be accounted for by changes in $Na_E + K_E$/TBW, hyperkalemia appear to result primarily from intercompartmental shifts of sodium and potassium.

External Secretory Function of Pancreas in Diabetes Mellitus. W. Y. CHEY, H. SHAY AND C. R. SHUMAN, Philadelphia, Pa. (introduced by Richard A. Kern).

Whether changes in external pancreatic secretion can occur in diabetes mellitus has long been debated. Responses of serum amylase and lipase and of the duodenal contents in the pancreozymin-secretin test were compared in 70 diabetic and 40 patients free of gastrointestinal disease. In 50 hospitalized adult diabetics selected by the method of randomization, 10 showed either elevated fasting serum enzyme values or an abnormal serum enzyme response after the hormones were injected and 18 patients showed abnormal values in the duodenal contents. Similar abnormal duodenal findings were obtained in all 6 cases of juvenile diabetes. The most common abnormality was a low amylase output. Diabetic patients with unexplained abdominal symptoms in whom other laboratory studies including gastrointestinal X-ray revealed no abnormality, yielded a higher incidence of abnormal results with the pancreozymin-secretin test than did those without such symptoms. Exocrine pancreatic function was further evaluated in selected patients by starch tolerance, I^{131}-triolein tests and stool examination for fat and undigested meat fiber. These results will be discussed. Duration or severity of disease or complications in the diabetics with abnormal external pancreatic function were not different from those with normal function. Involvement of the islets of Langerhans in primary chronic disease of the acini (chronic relapsing pancreatitis) is well known; the reverse process apparently also can occur in diabetes.

Demonstration of an Essential Role of the Anterior Pituitary in Cushing's Syndrome Associated with Bilateral Adrenal Cortical Hyperplasia. NICHOLAS P. CHRISTY* AND WILLIAM D. DRUCKER, New York, N. Y.

Many attempts have been made to detect adrenocorticotropin in blood of patients with Cushing's syndrome associated with bilateral adrenocortical hyperplasia in order to implicate the pituitary in etiology of the disease. Adrenocorticotropin has not been demonstrable except after adrenalectomy. Earlier work by Jailer and coworkers showed an adrenocorticotropin of obscure nature and origin in plasma of patients with the untreated disease. To characterize this adrenocorticotropin further, the observations have been extended using an accurate and sensitive bioassay: prevention of adrenal atrophy in hypophysectomized rats by 9-day injection of patients' plasma, endpoint being comparison between average adrenal weights (AAW) of groups of 6 to 8 injected rats vs those of 6 to 8 uninjected controls. In an untreated Addisonian, adrenocorticotropin was found: AAW of injected animals 9.5 mg $\pm$ SD 1.27 vs 7.5 ± 1.18, $p < 0.02$. In 4 patients with Cushing's syndrome associated with presumably autonomous adrenal tumor, adrenocorticotropin was never found: AAW 8.9 mg $\pm$ 0.66 vs 8.9 ± 1.14. In 11 of 13 patients with Cushing's syndrome associated with bilateral adrenocortical hyperplasia (without adrenocortical or pituitary tumor) adrenocorticotropin was detected: for the group, AAW was 10.8 mg $\pm$ 1.27 vs 8.3 ± 0.94, $p < 0.001$. Of significance was the finding in 3 such patients that adrenocorticotropin (AAW 11.2 mg $\pm$ 0.87 vs 8.7 ± 0.78, $p < 0.001$) disappeared after pituitary irradiation and clinical remission (AAW 10.3 mg $\pm$ 1.51 vs 9.6 ± 1.30, $p > 0.10$). The detection in Addison's disease of adrenal weight-maintaining activity suggests that this adrenocorticotropin is ACTH. Disappearance of adrenal weight-maintaining activity (conceivably ACTH) from plasma of patients with Cushing's syndrome associated with bilateral adrenocortical hyperplasia after successful pituitary irradiation suggests that the adrenocorticotropin is of pituitary origin, and that the pituitary plays an essential or primary role in maintaining the hyperadrenal state.

Experimental Staphylococcal Infection: The Role of α-Hemolysin and the Effect of Bacterial Endotoxin. LEIGHTON E. CLUFF* AND RICHARD C. CONTI, Baltimore, Md.

Experimental infection of normal animals and man with coagulase-positive staphylococci can be accomplished readily only by inoculation of large numbers of bacteria, indicating the high degree of natural resistance to infection by these microorganisms. For this reason it has been difficult to examine the mechanisms involved in production of host injury in stapyhlococcal disease. Bacterial endotoxin injected intradermally or intravenously in a pyrogenic but nonlethal dose induces in rabbits a striking increase in susceptibility to experimentally induced staphylococcal skin infection. This increased susceptibility is demonstrable only within the first 4 hours after injection of the endotoxin, and infection in endotoxin-prepared animals can be induced only with viable pathogenic staphylococci. The infection in endotoxin-prepared animals suggests that elaboration of α-hemolysin by multiplying staphylococci is responsible for production of hemorrhagic, necrotic and suppurative lesions. Immunity to α-hemolysin (active or passive) provides protection against infection by staphylococci in endotoxin-prepared rabbits. The effect of endotoxin upon staphylococcal infection cannot be inhibited with dibenamine, chlorpromazine or cortisone but is prevented by rendering rabbits tolerant to the pyrogenicity of the endotoxin. The critcial period during which endotoxin affects staphylococcal infection suggests that its action is mediated by an impairment of normal leukocyte function.

Metabolic and Hemodynamic Responses to Prolonged Upright Exercise: Observations on Trained and Untrained Men. LEONARD A. COBB, WILLARD P. JOHNSON AND ROBERT A. BRUCE,* Seattle, Wash.

Effects of walking at a steady, strenuous pace (3 mph, 18 per cent grade) were examined in 9 sedentary young men and in 3 well trained mountain climbers, 1 of whom was studied twice. Serial measurements of cardiac output (indicator dilution), heart rate, V_{O_2}, and arterial levels of lactate, pyruvate, glucose and free fatty acids were made while the subjects were exercising on a treadmill for periods of up to 75 minutes. Obvious fatigue was regularly observed in the untrained subjects. Excess lactate (XL) was used as an index of anaerobic metabolism (Huckabee). In 6 untrained subjects where XL was measured, it steadily rose to 5.5 to 7.5 μmoles per L at the termination of exercise. In mountain climbers, XL peaked at 4 to 6 minutes (1.0 to 2.3 μmoles per L) and fell to a low plateau through the remaining period of exercise. Plasma unesterified fatty acids fell initially in all subjects and rose in recovery; the highest "rebounds" occurred in the climbers (up to 1,950 μmoles per L). Heart rate during exercise increased steadily in all subjects and was consistently higher in the untrained men (+22 per minute). The average cardiac index during each of 3 temporally equivalent periods was higher in the 7 sedentary subjects (10.4 vs 9.3 L per minute per m^2). Calculated arteriovenous oxygen difference was significantly greater (+27 ml per L, $p < 0.001$) in the climbers, whereas there was no apparent difference in stroke index between the two groups (59 vs 60 ml per beat per m^2). During submaximal upright exercise, greater endurance for prolonged exertion in mountain climbers appears related to metabolic adaptations which permit more effective utilization of oxygen and prevent the accumulation of a large oxygen debt. Under these conditions, improvement of physical performance was not dependent upon enhancement of cardiac output or stroke volume.

Maturation of Liver Enzyme that Conjugates Sulfobromophthalein Sodium (BSP) and Glutathione. BURTON COMBES AND GENEVA SUE STAKELUM, Dallas, Tex. (introduced by Ralph Tompsett).

BSP removal from blood is delayed in premature and newborn infants soon after birth. Thereafter it gradually becomes normal. It has been suggested that this improvement in BSP removal from blood may be due to increased perfusion of the liver with blood and/or to maturation of hepatic function. The present studies were undertaken to determine whether this change in BSP removal might be related to alterations in BSP metabolism after birth. Recent studies have shown that the major pathway of BSP metabolism in several species involves intrahepatic conjugation with glutathione, a process catalyzed by a cytoplasmic enzyme. BSP metabolism was appraised, therefore, by measuring BSP-conjugating enzyme activity and glutathione levels (nitroprusside method of Grunert and Phillips) in livers obtained from rats *in utero* and at various periods after birth. BSP-conjugating enzyme activity, low prior to delivery, increased rapidly soon after birth and then gradually approached adult levels. Five and 3 days before the expected date of delivery, 0.15 mg BSP was conjugated per g liver per 5 minutes. This value was 0.4 mg 1 day prior to delivery, 0.5 to 0.6 mg 1 day postpartum, 1.25 mg at 3 to 4 days, 1.5 mg at 2 weeks, 2.0 mg at 5 weeks and 2.3 mg in adult rat liver. Mixing equal amounts of fetal and adult liver yielded values for enzyme activity equivalent to the arithmetic mean of fetal and adult activity, suggesting that low enzyme activity *in utero* was not due to the presence of an inhibitor in fetal liver, nor was increased activity related to the presence of an enzyme activator in adult liver. Glutathione levels in liver were also low prior to delivery, then gradually approach adult values. There was approximately 1.0 mg glutathione per g 2 to 3 days prior to delivery, 1.0 to 1.35 mg during the first and second weeks post partum, 1.5 mg at 3 weeks, then 1.5 to 2.0 mg during the fourth and subsequent weeks. The above data in the rat suggest that delayed BSP removal from blood soon after birth is related to impaired metabolism of BSP. Attainment of normal BSP removal could be

well correlated with maturation of BSP-conjugating enzyme and increased glutathione levels in liver.

Leukemic Cell Proliferation as Determined by In Vitro Deoxyribonucleic Acid (DNA) Synthesis. CHARLES G. CRADDOCK* AND GEORGE S. NAKAI, Los Angeles, Calif.

Assays of DNA synthesis by normal and leukemic cells (50 cases) were made under standard *in vitro* conditions employing both chemical extraction of P^{32} and tritiated thymidine (H^3T)-labeled DNA, as well as radioautography (H^3T). Points were obtained at 0.5 to 1 hour intervals for 3 hours and again at 19 hours for all parameters in selected instances. H^3T labeling was determined radioautographically both as per cent of labeled cells (per 600 cells) and the grain count distribution, counting immature cells only. The latter value was scored, points being assigned to each cell according to the intensity of labeling. The following observations were made: 1) The time course of H^3T uptake is quite different from that of P^{32} (or C^{14}-formate) in most cell systems; exogenous thymidine is rapidly polymerized with existing intracellular deoxynucleoside triphosphates of adenosine, guanosine and cytosine. This finding is consistent with the concept that endogenous thymidine formation lags behind that of the three other nucleosides. 2) In acute leukemic cells the pattern is often that of very low H^3T labeling (often not significantly above background by radioautography) by many cells. Here the chemical methods are necessary indices. 3) The patterns of labeling are quite distinctive for chronic myelogenous and chronic lymphocytic leukemias, where those cells which proliferate synthesize DNA at a rate comparable to normal myeloid and lymphoid precursors, respectively. The per cent of active cells in chronic lymphocytic leukemia is extremely small. The more blastic process tends to show less labeling on a per cell basis, especially in the lymphoblastic type. There are different patterns in the various types of acute leukemia. The evidence, when coupled with *in vivo* DNA labeling data, indicates a slow rate of proliferation per cell in some blastic leukemias, with a prolonged turnover of the leukemic tissue mass, and a prolonged cell survival. Alterations in the proliferative pattern during the progression of the disease will be described.

Idiopathic Hyperlipemia; the Behavior of the Isolated Chylomicrons as a Substrate for Lipoprotein Lipase. OSCAR B. CROFFORD, Nashville, Tenn. (introduced by Elliot V. Newman).

It is generally held that the rate-limiting step in the removal of chylomicrons from the plasma of patients with idiopathic hyperlipemia is the enzymatic hydrolysis of the plasma triglycerides. Some investigators have postulated an inherent abnormality in the chylomicrons which prevents their being readily attacked by the lipolytic enzymes. To study this possibility, comparisons were made between the isolated chylomicrons from a patient with idiopathic hyperlipemia and those isolated from a normal subject with postprandial lipemia. The comparative rates of lipolysis were determined by measuring the rate of glycerol production when the washed chylomicrons were incubated with lipoprotein lipase prepared from rat adipose tissue. The possibility of non-uniform affinity of the chylomicrons for the enzyme was explored by stepwise increases in the enzyme concentration, thus allowing the reaction to be carried to completion. It was demonstrated that the use of optical clearing as a quantitative index of triglyceride hydrolysis leads to serious errors if the substrate concentration is not adequately controlled. Optical clearing of an incubation mixture could be completely inhibited by the addition of nonturbid, triglyceride-rich plasma, even though lipolysis was proceeding at a linear rate. These results do not deny that optical clearing may be related to the degradation of chylomicrons which are large enough to scatter visible light, but if inequalities in the substrate concentration are produced by the addition of plasma which contains lipoproteins too small to cause visible turbidity, lipolysis can proceed at a maximal rate without visible clearing of the incubation mixture. The study shows that, with methods which are specific for lipolysis, there is no inherent abnormality of the chylomicrons which would explain the accumulation of triglycerides in the plasma of patients with idiopathic hyperlipemia and would place the metabolic defect at a site other than that which has been previously postulated.

Hemodynamic Effects of Oxygen Breathing. WALTER DALY AND STUART BONDURANT, Indianapolis, Ind. (introduced by John B. Hickam).

Despite common clinical usage of oxygen, conflicting data concerning its effects on cardiac index are available. An indocyanine green dilution method was used to evaluate this effect in 15 normal males. In random sequence, air or oxygen was inspired for 10 minutes before making observations. In 6 subjects, observations were made before and after release of arterial thigh tourniquets. In 7 other subjects, observations were made before and after atropinization. In nonatropinized subjects, oxygen breathing decreased the heart rate (69 ± 8 to 64 ± 9 per minute; $p < 0.001$), and cardiac index (3.01 ± 0.53 to 2.67 ± 0.46 L per minute per m^2, $p < 0.001$); stroke index remained unchanged (43 ± 7 ml per m^2); mean brachial pressure increased (93 ± 9 to 94 ± 8 mm Hg, p 0.005); systemic resistance increased (16.7 ± 3.1 to 18.1 ± 3.1 units, $p < 0.001$); circulation time increased (18 ± 3 to 21 ± 3 seconds, p 0.005). After atropinization, heart rate (96 to 97) and cardiac index (3.57 ± 0.48 to 3.50 ± 0.52 L per minute per m^2) were unaffected by oxygen; blood pressure increased (104 ± 8 to 108 ± 8 mm Hg, p 0.025); systemic resistance and circulation time were not affected. Arterial thigh tourniquet compression increased cardiac index

while air was breathed (2.77 ± 0.36 to 3.78 ± 0.38 L per minute per m^2, p 0.02); release of compression caused further increase to 4.59 ± 0.63 ($p < 0.01$). Thigh compression produced no increase during oxygen breathing, and release of compression produced an increase only to 3.74 ± 1.00 (p 0.01). Oxygen breathing (1 atm) produces a vagus-dependent decrease in heart rate and a rate-dependent decrease in cardiac index. In these observations, oxygen breathing abolished the effect of minor pain and anxiety on cardiac output and greatly decreased the effect of sudden release of arterial tourniquets. Oxygen effects must be considered in interpretation of physiologic data and are of obvious clinical significance.

The Effects of Antimitochondrial Sera on Mitochondrial Properties. JOHN S. DAVIS, IV AND ALFRED JAY BOLLET,* Charlottesville, Va.

Circulating factors which react with mitochondria and other cytoplasmic components have been reported in patients with systemic lupus erythematosus (SLE). The effects of these factors on properties of mitochondrial enzymes have been investigated and compared to those of antibodies produced in rabbits by injection of rat liver mitochondria in adjuvant. The rabbit antibodies and the lupus serum factors fixed complement with whole mitochondria, and with both the supernatant and sediment obtained after sonic disruption of mitochondria. The rabbit antisera caused precipitation of DPNH oxidase activity with sonicated mitochondria. Under appropriate circumstances, inhibition of DPNH oxidase activity of sonicated mitochondria by rabbit antisera could also be demonstrated. Sera from patients with SLE had a variable effect in these assays. The activity of these serum factors is being compared to the clinical status in the patients with SLE. The activity of several other mitochondrial enzymes was not inhibited by the antimitochondrial rabbit or lupus sera, including L-glutamic dehydrogenase, fumarase and "DPNH dehydrogenase," nor was acid phosphatase inhibited. However, antibodies produced by injection of rabbit with purified L-glutamic dehydrogenase did inhibit that enzyme in sonicated mitochondrial preparations. The rabbit antisera caused a release of L-glutamic dehydrogenase activity from isolated mitochondria into the supernatant solution, indicating an effect on the mitochondrial membrane. Sera from SLE patients had very little effect. The relative activity of various steps in the DPNH oxidase pathway in connective tissue harvested from polyvinyl sponges differed from the findings in liver. The DPNH-cytochrome C reductase/DPNH oxidase and "DPNH dehydrogenase"/DPNH oxidase ratios were considerably higher in the connective tissue. The influence of the antimitochondrial sera on the steps in the DPNH oxidase pathway and on ATP synthesis in different tissues is under study. These findings characterize some of the metabolic effects of antimitochondrial complement-fixing serum factors.

Fluorescence Spectrophotometric Studies of Metabolic Control in the Isolated Living Toad Bladder. ROBERT P. DAVIS AND MITZY CANESSA-FISCHER, New York, N. Y. (introduced by Quentin B. Deming).

Spectrophotometric studies of isolated mitochondria and unicellular organisms have revealed details of the processes of electron transfer and metabolic control. This is a report of the application of similar techniques to the study of active transport systems in an intact tissue in order to develop information on the metabolic regulation of transport. The fluorescence of reduced pyridine nucleotide (RPN), oxidized flavin nucleotides and tissue proteins was demonstrated in the living, intact, isolated toad bladder during and in the absence of active sodium transport. The quantitative response of the fluorescence intensities activated at 345, 325 and 290 mμ was observed in the resting state without added substrate, during transition from anaerobic to aerobic states, and after addition of glucose, adenosine diphosphate (ADP), pyruvate, succinate and amytal. Without disruption of biological structures, two fractions of RPN were demonstrated, one free and the other protein-bound. Study of these nucleotides under various conditions enabled identification of sites of inhibition of electron transfer and sites of coupling of energy changes of nucleotide reduction to phosphorylation of ADP. From the response to succinate, pyruvate and glucose addition, a succinoxidase system and a conventional pyridine nucleotide coupled electron transfer system were established. Very tight metabolic regulation by ADP has been demonstrated by the fall in fluorescent intensity of RPN following addition of ADP to the bladder. From the response to ADP, it is concluded that the bound RPN represents the special fraction of this nucleotide coupled to oxidative phosphorylation. The states of oxido-reduction of pyridine and flavin nucleotides in response to vasopressin have also been investigated. A marked increase in level of reduction and degree of protein-binding of nucleotides was shown. These data may provide information on the cellular biochemical mechanism by which energy is transduced to ion transport.

A Soluble Thyroidal Peroxidase-Iodinase System. LESLIE J. DEGROOT AND ANN M. DAVIS, Boston, Mass. (introduced by John B. Stanbury).

A soluble enzyme functioning as both iodide peroxidase and tyrosine iodinase has been extracted from sheep thyroids. It is released from mitochondria and microsomes by brief chymotrypsin digestion and extraction with N-butyl alcohol and acetone. It is nondialyzable, heat-labile, and is destroyed by prolonged enzymatic proteolysis. In standard conditions, enzyme (±200 μg protein), buffer, I^{131}, 10 mμmoles KI, 1 μmole tyrosine, 1 μmole glucose, and 5 mg glucose oxidase are incubated 30 minutes, with formation of 8 mμmoles iodotyrosine. Enzymic activity can also be followed spectrophotometrically as an increase in absorbance at the iodo-

tyrosine peaks. The enzyme iodinates free tyrosine, monoiodotyrosine and certain proteins, and requires a hydrogen peroxide source. Specific activity (mμmoles of iodide bound to tyrosine per mg protein) 500 times that of the starting material has been reached. A similar enzyme can be solubilized from salivary and splenic tissue, but not from liver, kidney, or brain. It has no reduced pyridine nucleotide oxidase activity, no iodotyrosine deiodinase activity, and no cytochrome oxidase activity. In striking contrast to the enzyme prior to solubilization from cell particles, it is inhibited by reduced pyridine nucleotide. The enzyme can be saturated, but not inhibited, by increased concentrations of iodide. It is inhibited by thiocyanate cyanide, but not by perchlorate. Methimazole is a strict competitive inhibitor. The ratio of diiodotyrosine to monoiodotyrosine formed varies from 0.1 to 0.7 and is a direct function of the molar ratio of iodine to tyrosine present in the incubation mixture. The enzyme shares many of the properties of thyroid tissue studied *in vivo*, and may be a part of the complete *in vivo* iodinating system. It appears to be a peroxidase-iodinase fragment, split away from the reduced pyridine nucleotide-flavin system which may serve as its *in vivo* H_2O_2 generating source.

The Importance of Autonomic Integrity in Maintaining the Hyperkinetic Circulatory Dynamics of Human Hyperthyroidism. WILLIAM J. DEGROOT, JAMES J. LEONARD, HYMAN W. PALEY, JOHN E. JOHNSON AND JAMES V. WARREN,* Galveston, Tex.

Increased responsiveness to sympathetic nerve activity and endogenous circulatory catecholamines have been implicated as important mediators of the hyperkinetic circulatory state seen in thyrotoxicosis. This hypothesis has been formulated primarily from *in vitro* and *in vivo* animal studies. The advent of pharmacologic agents capable of interrupting the integrity of the autonomic nervous system at various sites has provided a tool for the re-evaluation of this thesis in human hyperthyroidism. The response to three drugs was evaluated: 1) the ganglionic blocking agent, trimethaphan camphorsulfonate (Arfonad); 2) guanethidine, a postganglionic sympathetic neuroplegic; and 3) reserpine, an agent causing tissue catecholamine depletion. Dose and duration of therapy were individualized. A total of 19 hyperthyroid patients was studied (mean age 32) and each patient served as his own control. Cardiac output was measured by the Hamilton indicator dilution method. Heart rate, blood pressure, carotid pulse tracing and phonocardiogram were recorded with each cardiac output. Comparable normal data were obtained from 29 young volunteers. Trimethaphan camphorsulfonate caused no change in heart rate but reduced cardiac output by 11 per cent (5.3 to 4.7 L per minute per m^2; $p < 0.02$), thus remaining 57 per cent above normal (3.0 L per minute per m^2). Diminution of cardiac output was not effected by guanethidine (5.6 to 5.2 L per minute per m^2) or reserpine (5.8 to 6.2 L per minute per m^2). Guanethidine caused a pulse slowing of 20 per cent (109 to 89; $p < 0.01$) and reserpine a slowing of 17 per cent (102 to 85; $p < 0.01$). The isometric contraction period (0.02 second) and the duration of ventricular ejection (0.23 second), short in hyperthyroidism, were not normalized by these drugs. Chemical autonomic blockade with three pharmacologically different agents failed to modify the basic cardiovascular response to increased metabolic demands. A mechanism other than increased sensitivity to catecholamines appears to be responsible for the hyperkinetic circulation of clinical hyperthyroidism.

The Influence of Parathyroid Hormone on Bone Matrix Hexosamine Synthesis. WILLIAM P. DEISS,* LEILA B. HOLMES AND C. C. JOHNSTON, Indianapolis, Ind.

The close temporal relationship between calcium mobilization by parathyroid extract (PTE) and the disappearance of bone matrix hexosamine suggests that the action of parathyroid hormone (PTH) on bone leads to a disruption of both the mineral and organic phases, which are normally intimately associated. The present studies were designed to evaluate further the dynamic state of bone matrix hexosamine as influenced by PTH by measuring *in vivo* and *in vitro* incorporation of C^{14} from glucose into hexosamine of femur matrix. In rats injected with glucose-C^{14} 6.5 hours after pretreatment (75 U PTE or equivalent volume of control diluent every 2 hours for 3 doses), the specific activity curves of the serum hexosamine were nearly identical in the PTE and control groups. There was, however, a distinctly impaired incorporation of isotope into bone matrix hexosamine throughout the 12 hours following the glucose-C^{14}. The peak specific activity of the matrix hexosamine occurred 5 hours after isotope injection and at this time there was a decrease in matrix hexosamine concentration and a 34 per cent decrease in specific activity of the PTE group compared with controls. When the glucose-C^{14} was injected 20, 44, and 68 hours after PTE treatment, a stimulated matrix synthesis was manifested by a progressive increase in the peak specific activity of the matrix hexosamine (38, 53 and 69 per cent, respectively, above the controls) and by a return of the matrix hexosamine concentration to normal by 68 hours. *In vitro* incubation with glucose-C^{14} of minced femur from similarly pretreated rats also indicated increased incorporation of isotope into the matrix hexosamine of the PTH-treated group. These findings indicate an active turnover of bone matrix hexosamine. They also demonstrate a prompt suppression by PTH of matrix hexosamine synthesis accompanying calcium mobilization, followed by an intense compensatory resynthesis of organic matrix.

Dicumarol-induced Depression of a Thrombogenic Coagulation Activity in Man. DANIEL DEYKIN, STANFORD WESSLER,* STANLEY M. REIMER AND V. JANE CRANNEY, Boston, Mass.

We have recently described, in normal human blood, a thrombogenic coagulation activity (STA) that arises from an inactive precursor early in the elaboration of

"intrinsic" thromboplastin, is separable from all known procoagulants, and is measured by a quantitative bioassay. In the present study, assays of the STA activity of human sera, obtained from patients treated with bishydroxycoumarin (Dicumarol), were utilized as a reflection of the interference of this drug with *in vivo* thrombosis. In 7 patients on short-term and 13 patients on long-term therapy, the *in vitro* anticoagulant effects of Dicumarol were measured both by a modified Quick procedure and by the "thrombotest," whereas the *in vivo* antithrombotic effect was determined by the bioassay of STA activity. In addition, in the short-term subjects, serial *in vitro* assays of clotting factors II (prothrombin), VII (proconvertin), IX (PTC), and X (Stuart), and *in vivo* assays of STA activity were followed in all 7 patients together with appropriate controls. The sera of 6 of the 7 short-term patients demonstrated an antithrombotic effect that was observed in only 3 of the 13 long-term patients. In the short-term group the antithrombotic effect was independent of the depression of factors II, VII, IX, and X, singly or in combination, and, in several instances, did not appear until several days after the onset of the anticoagulant effect. We considered these data to represent the first experimental demonstration in human blood of an *in vivo* antithrombotic action of Dicumarol mediated through the intrinsic clotting system; they also indicate that Dicumarol depresses in human blood a hitherto unrecognized thrombogenic coagulation activity.

Left Ventricular Pressure-volume Loops in Man. HAROLD T. DODGE, HAROLD SANDLER AND ROBERT E. HAY, Seattle, Wash. (introduced by Robert S. Evans).

A method for determining left ventricular (LV) pressure-volume (P-V) loops in man has been developed and applied to study left ventricular hemodynamics in 12 subjects with mitral and/or aortic valvular disease. LV volumes (LVV) were calculated from biplane angiocardiograms taken at 6 per second and timed with respect to the cardiac cycle. Single composite volume curves were constructed from volume observations over several heart beats. P-V loops were constructed by relating LVV to LV pressure (LVP) recorded during (6 subjects) or just prior to angiocardiography. Individual LV P-V loops showed marked differences in magnitude and configuration, which were determined primarily by the type and severity of the valvular lesion. LV stroke work was calculated from the enclosed area, $\oint$ PdV, of the individual P-V loops and is the only method for calculating LV work in man with aortic and/or mitral valvular insufficiency. Stroke work varied from 55.9 to 457 g-m per beat. The location of individual P-V loops on the P-V axes varied widely and reflected the state of myocardial compensation. The area lying beneath the P-V loops, $\int_{V_S}^{V_D} P_D\,dV$, P_D = diastolic LVP, represented work done in distending the diastolic LV and shifts in the static level of pressure in the vascular system. With valvular insufficiency and heart failure, this area became large and was equivalent to work values as high as 79 g-m per beat. Under these conditions, a large error is introduced by methods which include this area in calculating LV work. LV P-V loops provide a previously unavailable method for calculating LV work in man with valvular insufficiency or mixed valvular lesions. This method does not require calculation of kinetic work in the aorta and permits differentiation of work done on the LV in diastole from work done by the ventricle in systole.

Malabsorption of Labeled Vitamin B_{12} in Rats with Intestinal Diverticula. R. M. DONALDSON, JR., Boston, Mass. (introduced by F. J. Ingelfinger).

Malabsorption of vitamin B_{12} by patients with the "blind-loop syndrome" is attributed to abnormally located intestinal bacteria, but exact mechanisms are unknown. E. W. Strauss and F. H. Gardner demonstrated malabsorption of labeled vitamin B_{12} in rats with mid-intestinal diverticula and showed that malabsorption was corrected by oral neomycin. Fecal excretion of radioactivity was used to measure Co^{60}-labeled vitamin B_{12} ($Co^{60}B_{12}$) absorption, and the results of Strauss and Gardner were confirmed. Absorption by rats with diverticula remained abnormal when $Co^{60}B_{12}$ was bound to neutralized rat gastric juice (RGJ). After surgical removal of intestinal pouches, $Co^{60}B_{12}$ absorption promptly increased to normal. A similar increase was noted when pouches were left in place but were surgically "by-passed" so that oral $Co^{60}B_{12}$ no longer entered the sacs. When isolated segments of ileum were perfused *in vivo* with $Co^{60}B_{12}$ bound to RGJ, intestinal uptake of radioactivity was the same in rats with diverticula as in control animals. Radioactivity was recovered from sac contents for 18 hours after oral $Co^{60}B_{12}$, and at 5 hours this amounted to 60 per cent of the dose. $Co^{60}B_{12}$ bound to RGJ was injected into pouches of untreated rats and rats fed neomycin. The precipitate of sac contents, thought to represent primarily bacteria and their products, was markedly reduced in amount in neomycin-fed rats. Furthermore, radioactivity recovered from precipitated contents was 76 to 94 per cent of the injected dose in untreated rats but never exceeded 25 per cent in neomycin-fed animals, and in treated rats the proportion of injected radioactivity found in pouch walls was greatly increased. These results suggest that malabsorption of labeled vitamin B_{12} in rats with diverticula is not due to bacterial elaboration of toxins or lack of intrinsic factor, but is more likely a consequence of uptake of $Co^{60}B_{12}$ by bacteria within the intestinal sac.

The Effect of Acetazolamide on the Secretion of Sodium and Potassium by the Human Stomach. D. A. DREILING, A. LINDER AND N. COHEN, New York, N. Y. (introduced by H. D. Janowitz).

The secretion of HCl by the mammalian stomach is dependent in part on the presence of carbonic anhydrase activity, as we have previously reported to this Society.

To obtain further evidence on the role of this enzyme in the gastric mucosa we have investigated the effects of the inhibitor acetazolamide on the secretion of Na and K by the human stomach. Gastric secretions were obtained by constant nasogastric suction in 20 subjects with a wide range of secretory activity. In 10, secretion was stimulated by histamine before and after the i.v. administration of acetazolamide (75 to 100 mg per kg) and analyzed for Na, K, Cl and H (titratable acidity to pH 3.5 and 7.0). Histamine augmented the rate of secretion of Na and K as well as demonstrating its expected increase in HCl secretion. Acetazolamide inhibited the output of Na and K as well as confirming the previously demonstrated reduction in acid output. The reduction of K and Cl was directly correlated with the reduction in rate of secretion. The reduction in H output was greater than could be accounted for by flow rates, and that of Na less; the well known inverse relation between Na and H concentrations was again observed. These results suggest that some Na enters the gastric juice in exchange for H, and that some K enters along with H. The effects of acetazolamide appear to be explicable in terms of the primary process of secretion and not necessarily on any subsequent process of exchange.

Early Effects of Vitamin A on Calcium, Citrate and Phosphorus Metabolism in Man. THEODORE DULL, PETER F. MAURICE, DOROTHY H. HENNEMAN AND PHILIP H. HENNEMAN,* Jersey City, N. J.

Mellanby, Wohlbach and others have demonstrated that vitamin A accelerates bone remodeling. Recently Clark has reported that vitamin A alters radiocalcium metabolism in the rat. Hypervitaminosis A in children produces painful periosteal new bone formation; in adults only acute toxic effects have been reported. We have administered vitamin A, 200,000 U daily for 3 to 4 weeks, during complete balance studies in 2 patients with hypoparathyroidism, 2 with sarcoid and hypercalcemia, 1 with presumed hyperparathyroidism and one normal subject. Vitamin A serum levels rose from 35 to 106 and from 65 to 176 μg per 100 ml in 2 subjects. Vitamin A markedly depressed serum and urinary citrate in all 6 subjects. Citrate changes preceded, were more marked than and persisted longer than the calcium and phosphorus changes. Serum calcium decreased from 10.9 to 9.1 mg per 100 ml in a patient with presumed hyperparathyroidism and decreased slightly in two other subjects. Urinary calcium fell significantly in 5 subjects; fecal calcium was unaltered. Urinary phosphorus decreased in 4 subjects; serum and fecal phosphorus did not change. Serum alkaline phosphatase and creatinine clearances were unchanged during vitamin A. Earliest effects were seen by 3 days, were maximal after 3 weeks and persisted for 1 to 3 weeks after discontinuation of vitamin A. Two subjects showing responses were hypoparathyroid. The phosphaturic effect of parathyroid extract and vitamin D enhancement of calcium absorption were unimpaired by vitamin A. Thus vitamin A in large doses in man appears to favor calcium and phosphorus retention by bone. These data do not indicate whether vitamin A stimulates osteogenesis or inhibits osteoclasis.

Demonstration of Tissue Antigens in the Blood Platelets. SHIRLEY EBBE AND MARIO BALDINI, Boston, Mass. (introduced by William Dameshek).

The previous finding in humans that repeated infusions of platelets from blood group-compatible donors reduced the viability of subsequently infused platelets raised the possibility that platelet antigenicity might be partially or totally dependent on transplantation antigens. To study this problem, 1) the survival time of skin homotransplants in rabbits previously sensitized to homologous platelets was determined, and 2) the viability of homologous platelets infused in rabbits sensitized by skin homografts was measured using platelets labeled *in vivo* with P^{32}. Skin homografts in rabbits sensitized by repeated intradermal injections of homologous platelets were rejected more rapidly than were homografts in nontreated controls. Skin homografts from the specific platelet donors had shorter survival than nonspecific homografts from other donors. Because platelet preparations used were not absolutely free of leukocytes, the reverse approach was then adopted. Viability of infused homologous platelets was measured in rabbits in which one to three consecutive skin homografts had previously been applied. After three grafts, viability of platelets from both specific and nonspecific donors was markedly reduced. Animals sensitized with one skin homotransplant followed by one platelet infusion showed strikingly reduced viability of subsequently infused homologous platelets, while a single skin homotransplant or a single platelet infusion did not appreciably change the viability of infused homologous platelets. This phenomenon had the characteristics of a secondary immunologic response to the applied skin homograft. Conclusions are that: 1) blood platelets and skin cells have antigens in common; 2) the common antigens are not strictly individual-specific; 3) it is possible that these platelet "tissue antigens" are transplantation antigens, and that measurement of platelet viability may become useful in the study of transplantation immunity; 4) these results imply that in thrombocytopenic bleeding repeated transfusions of blood platelets will have continued hemostatic effectiveness only in immunologically tolerant recipients.

Metabolism During Potassium Transport in Human Red Cells. ROBERT E. ECKEL* AND HARVEY LODISH, Cleveland, Ohio.

Cold-stored human blood accumulates fructose diphosphate (FDP) at the expense of inorganic phosphate (IP) and glucose. The red cells, washed in a saline-Tris buffer, and incubated with K (0.005 M), transport K in and Na out in the absence of added substrate for over 1 hour (transporting cells). In the absence of added K, the K concentration in the medium is about 0.001 M and no net transport occurs (resting cells). Intracellular

K concentrations are the same under the two conditions. Usually, but not always, transporting cells use more FDP. In all cases, transporting cells produce lactate and IP approximately equal to the FDP used. Resting cells produce about 20 per cent less of each. The fate of the remaining carbon and P in resting cells is not yet known. Several intermediates including pyruvate and lipid fractions have been excluded. Ouabain exaggerates the metabolic effects of hypokalemia and, in the presence of K, mimics the effects of hypokalemia. Nucleotide levels are low and little affected by experimental conditions. The following working hypotheses are suggested: 1) close coupling between glycolysis and ion transport exists in these experiments; 2) the increment in lactate and IP production in transporting cells represents a significant fraction of the metabolic cost of transport; 3) the diversion of carbon and P from these end-products demonstrates a control of intracellular metabolism by a mechanism (ion transport) involving extracellular ion concentration. That this change in metabolism is due to the arrest of transport and not to the hypokalemia per se is presumed from the similar metabolic effects of hypokalemia and ouabain and the assumption that ouabain is a specific inhibitor of transport.

Responses of the Peripheral Veins in Man to Continuous Positive Pressure Breathing. JOHN W. ECKSTEIN, A. W. HORSLEY AND WILLIAM K. HAMILTON, Iowa City, Iowa (introduced by E. L. DeGowin).

Venous pressure-volume curves were obtained from the right forearm of 8 healthy men, with a plethysmographic method. Venous pressure was measured in the left arm. Airway pressure was monitored through a needle inserted into a face mask. Observations were made with subjects breathing against ambient pressure and repeated when they inspired from and expired into a rubber reservoir bag. Outflow from the bag was restricted so that mask pressure remained positive during all respiratory phases. The flow of compressed air into the bag was sufficient to prevent serious elevation of CO_2 in the inspired air. Observations were made at a lower (average, 8.2 mm Hg) and higher (average, 15.0 mm Hg) level of mean mask pressure in each subject and repeated at the higher level 30 to 45 minutes following intravenous injection of morphine sulfate (15 mg). Venous pressure increases were roughly proportional to increases in mask pressure. Venous tone increased in 7 and remained unchanged in 1 of 8 subjects. In 4 the increase was sufficient to overcome the increase in venous pressure and prevent blood from pooling in the forearm. Pooling would have occurred in each case if venous tone had not increased. Forearm venous blood volume averaged 2.7 ml per 100 ml of tissue during control periods, 2.6 ml at the higher, and 2.6 ml at the lower mask pressure. The volume at the higher level would have averaged 3.2 ml if there had been only an increase in venous pressure and no increase in tone. Morphine did not prevent or modify significantly the venous response to breathing at the higher mask pressure. These observations suggest that increased venous tone may aid the return of blood to the heart during pressure breathing.

An Appraisal of the Venous Hematocrit. SEYMOUR EISENBERG, Dallas, Tex. (introduced by Ben Friedman).

The venous hematocrit has been criticized on the grounds that 1) it does not reflect the ratio of cells to plasma throughout the body, and 2) it entails errors due to "trapping" of plasma within the red cell layer and to incomplete packing of cells. To explore the validity of these criticisms, the following studies were performed. Utilizing an improved technic for plasma volume determination, the body hematocrit/venous hematocrit ratio was determined in 20 normal subjects; "plasma trapping" and incomplete packing were investigated with radioiodinated serum albumin-labeled plasma and microscopic examination of the cells. Labeled (I^{131}) polyvinylpyrrolidone (PVP-K90) was employed as a reference substance to measure plasma volume, and the red cell mass was simultaneously determined with Cr^{51}. The range of the body hematocrit/venous hematocrit ratio in 18 subjects was 90 to 105, with a mean of 96. Normal subjects were given radioalbumin (RISA, with <1 per cent free I^{131}). The radioactivity in whole blood and plasma sampled from these subjects was then determined and the ratio of cells to plasma calculated. Although these values agreed fairly well with corresponding Wintrobe hematocrit values, the latter were slightly lower than this calculated ratio (mean = 1 mm packed cell volume). The hematocrit tubes were then cut below the cell-plasma margin and the specific activity in suspensions of the packed cells determined; by this technic the amount of "trapped plasma" was consistently less than 1 per cent. Lastly, these cells were found to be markedly crenated. It is concluded that the venous hematocrit (Wintrobe) is an accurate measure of the ratio of cells to plasma. Previous estimates of the venous hematocrit/body hematocrit ratio have been falsely low. Minor errors due to slight trapping of plasma within the red cell layer are offset by crenation of the red cells.

Iron Uptake of Human Bone Marrow Incubated with Chloramphenicol. A. J. ERSLEV* AND I. IOSSIFIDES, Philadelphia, Pa.

Aplastic anemia is a well substantiated but rare complication of prolonged therapy with chloramphenicol. However, ferrokinetic studies have suggested that chloramphenicol quite commonly induces a reversible suppression of the erythropoietic tissue. In order to study the biochemical mechanism of this suppression and its possible relationship to aplastic anemia, human bone marrow obtained from patients undergoing rib resection was suspended in human plasma, divided into numerous aliquots, and the effect of chloramphenicol on the cellular uptake of Fe^{59} and total iron was determined after 4 and 21 hours' incubation. Chloramphenicol was found to induce a decrease in iron uptake directly

proportional to the concentration of drug with an uptake of 66 per cent of normal at a concentration of 400 μg per ml (about 15 times usual therapeutic levels). This decrease was probably not caused by a change in the rate of cellular multiplication since the difference between colchicinized and noncolchicinized samples was the same at all drug levels. The principle metabolic end-product of chloramphenicol, the biologically inactive glucuronide, was examined in the same system and found to be completely inert. However, three biologically inactive stereoisomers were found to be as "toxic" as chloramphenicol. The action on iron metabolism was related to the dichloroacetamide side chain rather than to the nitrobenzene group, since a naturally occurring hydrolytic product with a hydroxyacetamide side chain was inert. In order to gain a perspective of these findings relative to aplastic anemia, antibiotics not believed to cause aplastic anemia were also tested. Penicillin, streptomycin, kanamycin and vancomycin were found to be inert. However erythromycin was as "toxic" as chloramphenicol and all the tetracyclines were found to be even more "toxic," with iron uptake of 33 per cent of normal at a concentration of 100 μg per ml. The significance of these data will be discussed.

Urine Flow Response to Mannitol Loading in Unilateral Renal Disease. MELVIN H. FARMELANT AND BELTON A. BURROWS,* Boston, Mass.

External monitoring of individual kidney areas following intravenous injection of radioactive Diodrast or Hippuran yields curves whose fall-off rates are primarily a function of the urine flow rate. Dividing the ratio of the radioactivities existing in each kidney area at the time of peak uptake into the ratio present after an interval provides a percentage expression of the relative fall-off rates of the two curves for that interval. Such calculations were made in 27 hypertensive patients who showed a significant difference between the kidneys in urine flow rates by this technique. Comparisons were made between the effects of water loading and mannitol loading on urine flow rate differences (UFRD) in each patient. Four patients were similarly studied during ureteral catheterization. With water loading, 16 patients with renal arterial disease (proven by aortography, catheterization studies and surgical exploration) showed a greater mean UFRD than did the 11 patients with intrinsic or obstructive renal disease (47 vs 29 per cent, $p < 0.01$), with little overlapping of values. With mannitol diuresis, the mean UFRD in patients with arterial disease was reduced by 23 per cent, and in the intrinsic-obstructive group by 4 per cent, with no overlap in the percentage change in UFRD. The results with ureteral catheterization were comparable. Two patients with arterial lesions had a diminished UFRD and one showed no mannitol response during salt depletion, the expected UFRD and mannitol response being restored by salt repletion. These studies indicate that in man, as in the experimental animal, alterations in renal blood flow-pressure relationships result in marked reductions of urine flow, which can be partially restored by mannitol diuresis. Comparable UFRD's and mannitol responses were not observed in parenchymal or obstructive renal disease.

A New Protein Disturbance Associated with Rheumatoid Arthritis. RICHARD S. FARR, GERALD P. RODNAN AND ELLIOTT C. LASSER, Pittsburgh, Pa. (introduced by Jack D. Myers).

All human sera bind I^{131}-labeled sodium acetrizoate (I*Urokon) as measured by equilibrium dialysis performed at room temperature for 18 hours when the outside bath contained 0.8 per cent dextran in saline borate buffer at pH 7.4. When the concentration of unbound Urokon in solution at equilibrium was 73.8 μg per ml, a 0.95 per cent solution of human albumin bound 70.1 μg per ml and 1:5 dilutions of human sera from a variety of disease states, including rheumatoid arthritis, bound between 15.5 and 63.5 μg Urokon per ml. Under these conditions, the amount of Urokon bound was found to be related to albumin concentration. However, when the concentration of unbound I*Urokon at equilibrium was 0.06 μg per ml, 10 of 16 rheumatoid arthritis sera bound 2.7 to 4.3 times more Urokon than could be accounted for on the basis of albumin concentration. In contrast, the highest value obtained with 20 normal sera was 1.2×, with 15 scleroderma sera 2.5×, with 10 lupus erythematosus sera 1.9×, and with 38 sera from other acutely or chronically ill patients 2.4×. Human γ-globulin does not bind Urokon and the increased binding by rheumatoid sera did not correlate with globulin concentration or latex fixation reaction. Urokon binding to sera is highly specific in the sense that the addition of an acetylamino group at the fifth position in the benzene ring of Urokon prevents the interaction. Some other substituted benzoic molecules compete for Urokon binding sites, but increased binding by rheumatoid serum does not appear to be due to antisalicylate. These findings suggest that Urokon and related substances compete for specific receptor groups on normal albumin. Studies are in progress to determine whether increased Urokon binding associated with rheumatoid arthritis is due to an unusual albumin or to other peculiar proteins associated with the disease.

Experimental Reactivation of Subsiding Rheumatic Fever. ALVAN R. FEINSTEIN AND MARIO SPAGNUOLO, Irvington-on-Hudson, N. Y. (introduced by Currier McEwen).

The present work demonstrates the experimental provocation of rheumatic activity in asymptomatic patients and provides an explanation for the mechanism of the post-therapeutic rebound phenomenon in rheumatic fever. A 2-week course of treatment with prednisone, aspirin, or nothing was given experimentally to 88 randomly allocated patients convalescing from rheumatic fever. The experimental treatment in each patient was begun exactly 1 month after discontinuation of the initial course of treatment, a time when all patients had no *clinical* evidence of rheumatic activity. During retreatment, the

sedimentation rate and C-reactive protein were invariably lowered by prednisone, but were sometimes raised by aspirin. The mean hematocrit rose with prednisone and fell with aspirin. When the experimental therapy was stopped, a recrudescence of fever, tachycardia, arthralgia, erythema marginatum, or congestive heart failure occurred in 9 of 29 patients treated with prednisone and in 1 of 29 patients treated with aspirin. The 30 controls remained asymptomatic. The experimental rebounds were more frequent in patients with severe cardiac damage and occurred only in patients who had shown clinical or laboratory evidence of rheumatic activity immediately after their initial course of treatment. These results contradict the hypotheses that the rheumatic rebound is due to either 1) polycyclic rheumatic activity, 2) relative hypoadrenalism, or 3) insufficient duration of therapy. They indicate that the rebound represents the appearance of rheumatic inflammation which accumulated under therapeutic suppression. They also indicate that anti-inflammatory therapy can be detrimental for asymptomatic rheumatic patients whose only abnormalities are in laboratory tests.

Granulocyte Response to Endotoxin in Myeloproliferative Disorders. MARTIN E. FINK, PAUL CALABRESI AND STUART C. FINCH,* New Haven, Conn.

Studies were performed to determine the magnitude and characteristics of the leukocyte response to endotoxin in patients with myeloproliferative disorders. Sixteen patients without hematological abnormalities, 4 with chronic granulocytic leukemia (CGL), 4 with myelofibrosis and 2 with polycythemia vera received injections of Pyrexal, 0.1 μg intravenously. Total and differential leukocyte counts were performed before injection and at hourly intervals for 7 hours. The properties of the mobilized granulocytes in the myeloproliferative group were also compared with four controls with respect to hourly changes in 1) enzyme content (alkaline phosphatase), 2) cytoplasmic integrity (lysozyme release), 3) participation in the local inflammatory response (Rebuck technique), and 4) phagocytic capacity (sensitized starch granules). The typical response was a granulocytic leukocytosis reaching its peak in 3 to 5 hours. The average absolute granulocyte increase in the normals was 6,590 cells per mm^3 (range, 2,220 to 12,393) and consisted only of mature cells compared to 21,200 mature granulocytes per mm^3 (range, 12,740 to 29,880) and 2,776 immature forms per mm^3 (range, 1,690 to 4,389) in patients with CGL, although their disease was well controlled with busulfan. In three patients with classical myelofibrosis, increases of only 978 granulocytes per mm^3 (range, 0 to 1,478) were obtained. The fourth patient whose baseline leukocyte count was 62,000 and, unlike the others, had no elevation of granulocyte alkaline phosphatase, manifested a normal granulocyte response, as did the subjects with polycythemia. A slight but consistent elevation of the granulocyte alkaline phosphatase score was observed at 1 to 2 hours. A significant rise in serum lysozyme level was detected only in one patient with CGL following a peak response of 27,990 granulocytes per mm^3. In all cases, mobilized granulocytes manifested no change in their ability to participate in the local inflammatory response or in their capacity to phagocytize sensitized starch granules.

The Course of Urinary Tract Infections Secondary to Catheterizations. JANET J. FISCHER, Chapel Hill, N. C. (introduced by Louis G. Welt).

One hundred ninety-four patients hospitalized on the Gynecology Service were studied and followed to determine the incidence of, and the course of urinary tract infections in this group over a 2 year period. The vast majority of these patients had carcinoma, usually of the cervix, but some had carcinoma of the uterus or of the vagina. Ninety per cent of the patients were catheterized and 40 per cent had an indwelling catheter in place for at least 2 days. At the time of admission to the study, 85 per cent were uninfected. Two to 4 weeks later, 35 per cent (26 patients) of these uninfected patients subjected to one catheterization, 25 per cent (2 patients) of those not catheterized at all, and 60 per cent (34 patients) of those with indwelling catheters were infected. Ninety per cent of the infections were due to gram-negative rods, 45 per cent to *Escherichia coli*, and 25 per cent to Aerobacter-Klebsiella group. At 3 months, 50 per cent of the patients with *E. coli* still were infected. Repeated infections with different organisms were present in 3 per cent of the patients. Contrary to the accepted concept of the course of carcinoma in these patients, analysis of these data support the idea that the occurrence of urinary tract infections in this group of patients is related to catheterizations performed as part of therapy, and that some of these patients manifest uremia as a result of progressive urinary tract infection before urinary tract obstruction has occurred.

The Diagnosis and Quantitative Evaluation of Mitral Regurgitation. CHARLES W. FRANK, FRIDO H. KIEFHABER, ALPHONZO JORDAN, TURHAN AKYOL, BARRY B. GALTON AND YOGESH ARORA, New York, N. Y. (introduced by Howard A. Eder).

It has long been appreciated that mitral regurgitation can be recognized by the appearance of an indicator dye within the left atrium immediately after its introduction into the left ventricle. We have found that regurgitation induces a specific systemic arterial disappearance pattern following ventricular injection. The normal single exponential rate of disappearance is initially accelerated (while atrial dye content is increasing), and finally retarded (while the mixed atrioventricular volumes are washed out together). The volume of the regurgitant flow may be calculated from simultaneous left atrial and systemic arterial dye dilution curves following ventricular injection if certain conditions prevail. One is that the left atrial sample accurately represents the blood-dye mixture which re-enters the left ventricle from the atrium, in which case the rate at which dye disappears from the atrial sampling site will be identical to the final

rate of disappearance from the ventricle. This same rate should also be exhibited after injection into the atrium. Left atrial and systemic arterial dye dilution curves were obtained from 21 patients with rheumatic heart disease following injections of indocyanine green into the left ventricle and left atrium. Ventricular injection was made through a catheter designed to promote an initial even dispersion of dye within the ventricle. Left atrial sampling and injections were performed through a Ross needle. Ventricular disappearance rates were estimated from systemic arterial dilution curves. Mitral regurgitation was detected in 10 patients by atrial sampling. In 8 of these, the disappearance rate from the atrium following ventricular injection was similar to the final rate from the systemic artery, and to the arterial disappearance rate following atrial injection. This indicates that atrial mixing and sampling conditions were adequate to permit quantitative measurement of regurgitant flow.

The Initial Step in Active Sodium Transport by the Toad Bladder. HOWARD S. FRAZIER, ELEANOR F. DEMPSEY AND ALEXANDER LEAF,* Boston, Mass.

Movement of sodium from bladder epithelial cell interior to serosal medium occurs by active transport. Since net sodium transport continues when as little as 0.1 mEq of sodium is present in mucosal medium, studies were undertaken to learn how sodium moved across the mucosal surface. Electrical forces at the mucosal surface were measured with intracellular glass microelectrodes. The potential was measured at 114 or <0.2 mEq sodium per L of mucosal medium. In the spontaneously active state the cell interior was always electrically positive to the mucosal medium. In the short-circuited condition the cell interior was at the same potential or very slightly negative to it. Therefore electrical driving forces do not explain net entry of sodium from low sodium media. The concentration forces were evaluated by altering sodium concentration in the mucosal medium and measuring isotopically the pool of cellular sodium which derived from the sodium in the mucosal medium. The results were consistent with the interpretation that the concentration of sodium in the cell water was always less than that in the mucosal medium and hence that a concentration gradient could account for mucosal entry of sodium. The short-circuit current as a function of mucosal concentration of sodium showed saturation kinetics. This could result from saturation either of mucosal entry or serosal exit of sodium from the cell. Measurements of the cellular sodium pool showed that entry was rate-limiting, and although passive, not by free diffusion. Vasopressin increased the active transport of sodium at any mucosal concentration of sodium by increasing mucosal permeability to sodium and hence the availability of sodium at the serosal pumping site. This site of the hormonal action is the same as that previously demonstrated for urea and water. It is unnecessary to postulate a direct effect of the hormone on the sodium pump.

Response to Repeated Platelet Transfusions from the Same Donor. EMIL J. FREIREICH, ALLAN KLIMAN, LAWRENCE A. GAYDOS AND LESLIE R. SCHROEDER, Bethesda, Md. (introduced by C. Gordon Zubrod).

Development of effective platelet replacement therapy has been hampered by inadequate supplies of fresh platelets and by the anticipation of progressive resistance to repeated transfusions. We have studied the response to repeated platelet transfusions from a single donor by utilizing plasmapheresis, a technique which increases at least 32-fold the quantity of fresh platelets that a donor can supply. Platelet-rich plasma (500 ml) obtained by plasmapheresis of a single donor was administered within 6 hours of collection to 28 children with severe thrombocytopenia secondary to acute leukemia. A significant increase in circulating platelet count with progressive fall to pretreatment levels was observed in 26 patients. Platelets were transfused as often as 3 times per week. Twenty patients received 2 or more treatments at least 7 days apart from a single donor (median—6 treatments per patient, range 2 to 19). Increase in circulating platelet count and rate of decline to pretreatment levels remained comparable throughout courses of repeated platelet transfusions of 8 to 365 days (median 25). Control of gross hemorrhage, correlated with increase of circulating platelets, was repeatedly observed. Thus resistance to repeated platelet transfusions from the same donor could not be demonstrated. Plasmapheresis of blood donors to provide platelet-rich plasma in large quantities proved a repeatedly effective method of platelet replacement therapy and offers a means of controlling donor factors in platelet transfusion studies.

The Origin of Fasting Plasma Triglycerides in Man. SAMUEL J. FRIEDBERG, ROBERT F. KLEIN, DAVID L. TROUT, MORTON D. BOGDONOFF AND E. HARVEY ESTES, JR., Durham, N. C. (introduced by David T. Smith).

Available evidence indicates that a fraction of intravenously administered palmitic acid-1-C^{14} appears as C^{14}-labeled plasma triglycerides (TG). In order to assess the quantitative significance of the conversion of plasma free fatty acids (FFA) to plasma TG in man, C^{14}-labeled palmitic, oleic and stearic acids were administered intravenously to 13 fasting male subjects without known disorders of fat metabolism. Thereafter venous blood was sampled at appropriate and frequent intervals over a period of 24 hours. The following were determined: total plasma FFA and plasma TG; also, activities of plasma TG, cholesterol, cholesterol esters, phospholipids and FFA. Plasma lipids were separated by silicic acid chromatography and by chemical methods. It was found that radioactivity appeared almost exclusively in triglycerides during the first 6 hours and that the disappearance of triglyceride during this period was first-order in type. Later, activity appeared in free cholesterol, cholesterol esters and phospholipids. From these determinations the following data were derived: 1) the individual plasma FFA turnover rates, 2) plasma

TG turnover rates, and 3) the fraction of administered labeled fatty acid initially equilibrating into plasma as labeled TG. From the fractional turnover rate of fasting plasma TG, which ranged in normals from 10 to 35 per cent per hour, and from the other data it was estimated that plasma FFA are the major source of plasma TG and that all three triglyceride fatty acids turn over. It was also found that the fractional turnover rates for triglyceride fatty acid were the same for oleic, stearic, and palmitic acids, but that relatively less stearic acid appeared in TG. The anticipated level of plasma triglycerides derived from the tracer studies agreed well with the observed plasma TG concentration.

Nonspecificity of Human Skin Graft Rejection. ELI A. FRIEDMAN, J. WALDEN RETAN, DAVID C. MARSHALL, LAWRENCE B. HENRY AND JOHN P. MERRILL,* Boston, Mass.

Previous studies of mammalian homograft rejection emphasized the individual specificity of the transplantation antigen(s). Berrian and Jacobs have recently shown that mice preimmunized with homologous spleen cells subsequently reject the skin of the splenic cell donor or a nonspecific donor in a pattern dependent upon the number of shared transplantation antigens. Attempts to immunize humans with leukocytes harvested from peripheral venous blood by a dextran sedimentation technique have been unsuccessful via either intravenous or subcutaneous routes. Sixteen ambulatory adults were "immunized" with 2 to 30×10^7 leukocytes injected intracutaneously at 30 different sites 6 days prior to challenge grafting. Full-thickness skin homografts taken from unrelated individuals and from the leukocyte donor were placed on the recipient's arm. Serial gross observation and microscopic examination of periodic graft biopsies were compared with control unmodified rejections in other patients. Leukocyte donor skin was rejected as a "white graft" (hyperimmune state) in 8 instances, and as an accelerated rejection in 7. Of 14 simultaneously placed grafts from nonleukocyte donors, 4 were rejected as white grafts and 6 as accelerated rejections. The type of rejection encountered was partly contingent upon the number of immunizing leukocytes employed. In this human system transplantation immunity was not entirely individual-specific. The technique suggests a method by which diversity or similarity of transplantation antigens in the prospective human transplant donor and recipient might be explored.

Cystinuria: Amino Acid Clearances and the Occurrence of a Cystine Peptide. GEORGE W. FRIMPTER, AUDREY BASS, EUGENE FURTH, MELVIN HORWITH AND DAVID D. THOMPSON,* New York, N. Y.

Renal excretion of amino acids was studied in 9 patients with cystinuria. Ion exchange chromatography with an automatic apparatus was used for identification and quantitation of amino acids. In 4 patients inulin and endogenous amino acid clearances were measured simultaneously. Reduced reabsorption of cystine, ornithine, lysine and arginine was observed in all patients. In all of the urinary ion exchange chromatograms, a previously undescribed compound was demonstrated. This substance was not found in urine from normal subjects or from patients with pyelonephritis, nephrosis, metabolic conditions with stone formation, cystinosis, or other amino acidurias. The compound was not detected in plasma of normal subjects or patients with cystinuria. It was not found following incubation of normal urine with cystine, cysteine or methionine, or in urine of normal subjects after ingestion of these amino acids, or in urine of a normal dog during massive cystine excretion due to intravenous cysteine loading. The compound *was* present in urine of a dog with cystinuria and cystine stones, but was *not* found in urine of a Kenya genet, a species that uniformly exhibits cystinuria but does not form cystine stones. Traces of the substance were found in stones from patients with cystinuria. The compound was isolated by a series of chromatographic separations using various ion exchange resins. Following these procedures the compound was shown by chromatography to produce a single peak, with an Rf identical to that noted originally. The purified compound gave positive tests for peptide, and upon hydrolysis yielded a large amount of cystine plus other amino acids. A rapid escape through cellophane suggested a low molecular weight. The possible relationship of this compound to cystinuria is considered.

The Effects of Guanethidine on Triiodothyronine-induced Hyperthyroidism in Man. THOMAS E. GAFFNEY AND EUGENE BRAUNWALD, Bethesda, Md. (introduced by Robert W. Berliner).

This investigation, designed to assess the contribution of the sympathetic nervous system to the human hyperthyroid state, was carried out because it has been suspected that many of the manifestations of hyperthyroidism are mediated or potentiated by the sympatho-adrenal system. In 4 normal subjects triiodothyronine was administered in daily doses of 250 to 350 μg and resulted in clear-cut signs and symptoms of hyperthyroidism. The heart rates during sleep rose to levels 35 to 55 per cent above control, basal metabolic rates rose to levels 25 to 50 per cent above control, and serum cholesterol fell by 75 to 105 mg%. Palpitations and tremor characteristic of hyperthroidism also developed in all subjects. Guanethidine, a drug which selectively interrupts adrenergic reflexes and produces tissue catecholamine depletion, was then administered in daily doses of 20 to 50 mg, while triiodothyronine was continued. In all 4 subjects the heart rate returned to control levels, the basal metabolic rate fell to 18 to 23 per cent above control levels; palpitations and tremor disappeared, but serum cholesterol remained depressed. When guanethidine was withdrawn the tachycardia, hypermetabolism, palpitations and tremor returned. These observations indicate that the tachycardia, hypermetabolism, and tremor of hyperthyroidism are intimately related to

activity of the adrenergic nervous system and/or tissue catecholamine content; the cholesterol-depressing effect of triiodothyronine appears to be unrelated to these factors. It is suggested that guanethidine may be of clinical value in rapidly counteracting many of the manifestations of the hyperthyroid state. Pharmacologic inhibition of the adrenergic system may also be useful when thyroid hormones or their analogues are administered in order to depress serum cholesterol.

The Survival of Virus-treated Erythrocytes in Normal and Splenectomized Rabbits. EDWARD GARDNER, JR., CLAUDE-STARR WRIGHT* AND BETTIE Z. WILLIAMS, Augusta, Ga.

The survival of virus-treated erythrocytes in normal and splenectomized rabbits was studied using radiochromium (Cr^{51}) labeled cells. This investigation is a part of a long-range program attempting to correlate destruction of the damaged or modified red blood cell and the spleen. Erythrocytes were labeled with radiochromium (Cr^{51}) using *in vitro* and *in vivo* technics. The survival of normal erythrocytes in normal animals was the same by both technics. Normal erythrocytes in normal rabbits showed a mean apparent half-time survival of 12 days. Intersection of the survival curve with the time axis occurred between 55 and 60 days for those studies carried to completion. Normal erythrocytes in splenectomized animals showed a half-time survival at the upper limits of normal. Erythrocytes treated with influenza and Newcastle disease virus showed a shortened survival time in normal rabbits. Newcastle disease virus apparently produced more damage to the red blood cells than influenza virus as judged by the markedly shortened survival of cells treated with this agent. Erythrocytes treated with influenza virus appeared to survive longer in rabbits previously splenectomized. These findings suggest that the removal of this source of reticuloendothelial tissue prevented a more rapid removal of the virus-modified cells from the general circulation.

Stimulatory Effect of Phenobarbital on Steroid Metabolism. LEONARD D. GARREN, A. H. CONNEY AND GORDON M. TOMKINS,* Bethesda, Md. and Tuckahoe, N. Y.

Administration of phenobarbital and other drugs greatly increases the activity of several drug-metabolizing enzymes which are localized in liver microsomes and which require reduced triphosphopyridine nucleotide (TPNH) for activity. Since several steroid-metabolizing enzymes resemble the drug systems in their intracellular distribution and cofactor requirements, the effect of phenobarbital administration on the enzymic metabolism of steroids by rat liver microsomes was investigated. It was observed that microsomes from 40-g rats which had been injected with phenobarbital (75 mg per kg) for several days catalyzed a rapid oxidation of TPNH in the presence of androsterone (or 4-androstene-3,17-dione). Microsomes from untreated controls did not catalyze such a reaction. Partition chromatography of the products of androsterone metabolism revealed a single compound, more polar than the substrate, which possessed an unaltered keto-group at C-17. Its infrared spectrum suggested that it was a hydroxylated derivative of androsterone. The spectrum was not consistent with a 2-hydroxy compound and paper chromatographic studies showed that 11β-hydroxy-androsterone was not the product. Further studies are in progress in order to identify the unknown steroid. Thus, administration of a foreign compound, phenobarbital, influenced the metabolism of normally occurring steroids by apparently stimulating their hydroxylation by a TPNH-dependent microsomal enzyme.

The Role of Activation Product in the Thromboplastin Generation Test. LAMONT W. GASTON AND HELEN DEORSAY, Boston, Mass. (introduced by Kurt J. Isselbacher).

Serum obtained from blood incubated for 24 hours at 37° C supports normal generation of thromboplastin in the thromboplastin generation test (TGT), but there is a delay of 5 to 6 minutes before maximal activity is reached. If a sample of the serum is then kept in a glass tube, there is a progressive increase in the rate but not the amount of thromboplastin formation. If kept in a siliconed tube, however, the TGT curve is unaltered. Using the activation product (AP) assay of Waaler which measures the combined activity of Hageman factor and plasma thromboplastin antecedent (PTA), we observed that the increased rate of thromboplastin formation following exposure of the serum to glass was paralleled by a rising level of AP with no accompanying change in the Stuart factor level. The effect of glass contact (i.e., increase in rate but not quantity of thromboplastin formation) was exactly duplicated by addition to a TGT incubation mixture containing serum from a siliconed tube of a preparation of AP containing no plasma thromboplastin component (PTC) and only a trace of Stuart factor. The rate of thromboplastin formation, as distinct from the amount formed, has been attributed to a "Factor X" or, more recently, to prephase accelerator. It seems likely, however, that rate of formation is controlled by AP. Still another serum factor other than Stuart factor, PTC, and AP may also affect the TGT. The serum of a patient with Hodgkin's lymphoma performed abnormally in the TGT yet contained normal amounts of Stuart factor and PTA and showed the glass contact phenomenon described above.

Asynchronous Thymidine Uptake by Human Chromosomes. JAMES L. GERMAN, III, New York, N. Y. (introduced by Alexander G. Bearn).

Chromosomes of germinal and somatic cells of plants and lower animals may undergo asynchronous replication of deoxyribonucleic acid. Of special interest is the

demonstration by Taylor that in somatic cells of hamster the sex chromosomes and certain other chromosomes of the smallest order may be relatively late in undergoing chromatid reproduction during interkinesis. It is undetermined whether a similar sequence occurs in germinal cells or whether the phenomenon is of importance in relation to the frequency of non-disjunction of chromosomes at meiosis. Human nucleated blood cells of 2 males and 1 female, freshly isolated from the individual using phytohemagglutinin, were placed in suspension tissue cultures. After 3 days *in vitro*, cells undergoing division were accumulated in metaphase by 30-minute exposure to desacetyl-methyl-colchicine. At various intervals preceding this metaphase arrest, different cultures were placed in a medium containing tritium-labeled thymidine for a 10-minute period. Radioactivity of individual chromosomes was determined by radioautography. Tritium uptake, indicating DNA synthesis, was observed in as many as 4 per cent of both large and small round cell nuclei. In 7 experiments examination of 1,369 metaphase figures indicated that thymidine incorporation had occurred in 95 per cent of such cells in the time period of 30 to 4 hours preceding metaphase. Cells reaching metaphase which had been exposed to labeled thymidine shortly before or after this period showed relatively little radioactivity. Essentially no uptake occurred during the 2 hours immediately preceding metaphase. Furthermore, label uptake was asynchronous, some chromosomes of the complement incorporating thymidine when exposed to tritium-labeled thymidine at a given time before mitosis while others remained nonradioactive. Therefore, cells cultured from venous blood 1) display a cycle in excess of 30 hours; 2) some chromosomes are later than others in completing thymidine incorporation.

Release of Glutamic-oxalacetic Transaminase in Virus-infected Tissue Cultures. V. E. Gilbert, R. Pavia and A. I. Braude,* Pittsburgh, Pa.

Elevation of serum glutamic-oxalacetic transaminase (GOT) in viral hepatitis suggests that viruses release this enzyme from cells. In order to determine whether GOT is released from viral infected cells, GOT was assayed in tissue cultures infected with various cytopathogenic viruses. Spectrophotometric measurement disclosed sharp rises of GOT in fluids of stationary cultures of monkey kidney (MK), human embryo kidney (HEK), HeLa and human amnion (FL) upon infection with all enteroviruses examined. Echo 9 (MK, HEK), 7 and 12 (MK) and Coxsackie B3 (MK, HEK, HeLa, FL) and B5 (MK, HeLa, FL) released GOT in proportion to the degree of gross cell destruction. GOT was not released until cytopathogenicity progressed beyond early stages despite early virus release. Peak values of 50 to 169 U per ml per minute were reached coincident with the disappearance of most swollen cells. Complete destruction of MK cell sheets by Echo 12 released only half of total cellular GOT from these infected cells. This suggests that many cells resist infection or lysis. Unlike enteroviruses, adenovirus type 7 failed to release GOT upon infection of MK, HeLa, and FL despite severe cytopathogenicity; and only slight elevations occurred when the sheet left the glass. These findings coincide with the fact that cells infected with adenoviruses characteristically resist lysis and release only few viral particles. In preliminary studies with this adenovirus, total cellular GOT remained below that of uninfected MK cultures. In MK cultures infected with Ca (croup-associated) virus GOT was slowly released in quantities comparable to enteroviruses but without discernible cytolysis. Herpes simplex infection of MK and HEK cultures released only low levels of GOT as marked cytopathogenic changes developed. These studies indicate that GOT is released from cells of diverse origin by certain cytopathogenic viruses but not by other viruses capable of different types of cell injury.

The Antigenicity of Synthetic Polypeptides. Thomas J. Gill, III and Paul Doty, Cambridge, Mass. (introduced by Gustave J. Dammin).

Linear chain synthetic polypeptides were studied for their ability to elicit precipitating antibody formation and skin reactivity in rabbits. The metabolism of a radioiodinated polypeptide was also followed. The polypeptides had a molecular weight range of 100,000 to 200,000 and contained glutamic acid, lysine, tyrosine and phenylalanine in different combinations. The polypeptides containing glutamic acid and lysine (3:2) plus 5.8 per cent tyrosine and 8.7 per cent phenylalanine elicited an average antibody titer of 208 and 80 μg Ab N per ml serum, respectively; these were the highest titers observed. Copoly-glutamic acid-lysine (3:2) elicited an average antibody titer of 70 μg Ab N per ml serum. Rabbits sensitized with each of these three polypeptides developed Arthus reactions in skin test sites. Polyglutamic acid and polylysine were not immunogenic. Thus, copoly-glutamic acid-lysine was immunogenic while the homopolymers were not. Aromatic amino acids are not necessary for immunogenicity, but antigens containing them elicit higher antibody titers. A polypeptide containing glutamic acid and lysine (3:2) plus 1.3 per cent tyrosine elicited an average antibody of only 18 μg Ab N per ml serum and produced a skin reaction with a delayed time course. These synthetic antigen-antibody systems behaved like native protein systems with respect to the effects of dilution, salt concentration and time on the formation of the specific precipitate, but were somewhat more sensitive to temperature and pH variations. The molar antibody:antigen ratios for these synthetic antigens were 3 to 4 times higher than those found with native proteins of the same size. The physicocochemical and biological behavior of these synthetic polypeptide antigens showed them to be good protein models in an immunological system. The synthetic control of structure offers many possibilities to study the effect of a single parameter in the antigen on the nature of the immune response.

Auto-immunity in Liver Disease. Serological Investigations and Clinical Correlation. MUHARREM GÖKCEN, Minneapolis, Minn. (introduced by F. W. Hoffbauer).

The presence of circulating autoantibodies against human liver antigens has been sought by means of a modified complement fixation test in 171 patients with various types of liver disease. The liver autoantibodies have been demonstrated in all active cases of primary biliary cirrhosis, "postnecrotic" cirrhosis, "plasma cell hepatitis," "lupoid hepatitis," drug-induced jaundice, and in some cases of subacute lupus. The autoantibodies of primary biliary cirrhosis appeared to be distinct from those of postnecrotic cirrhosis in terms of reacting with different liver antigens. One hundred thirty-two different sera from blood bank donors served as controls and were screened with different liver antigens; none gave a positive titer. The autoantibody titer was well correlated with the severity of the disease process as well as with the amount of serum γ-globulin in which the autoantibodies resided. With spontaneous or steroid-induced remission, the serum autoantibodies either declined significantly or disappeared. The autoantibodies were lacking in a case of agammaglobulinemia with active postnecrotic cirrhosis. A titer of 1:128 was noted in 1 of 15 patients with acute hepatitis; 14 were negative. One case prolonged for 3 months developed a high titer of postnecrotic type antibodies. Cases of Laennec's cirrhosis did not have complement-fixing type antibodies but had the hemagglutinin type in low titer. Immunelectrophoretic studies were done in 20 patients selected from among the above 171 cases. A heavy β_2 M globulin (macroglobulin) band was observed in all 3 cases of Laennec's cirrhosis, 5 postnecrotic cirrhosis, 3 primary biliary cirrhosis, 1 lupoid hepatitis and 1 plasma cell hepatitis. This was but faintly present in 20 normal individuals and in 7 patients with jaundice, including 3 with biliary obstruction, 1 ulcerative colitis, 1 drug (PAS-INH)-induced, 1 recurrent jaundice of pregnancy, and 1 of plasma cell hepatitis in steroid-induced remission.

A Study of the Patterns of Solute Excretion in Acute Renal Failure in Relation to the Duration and Pathogenesis of Oliguria. MARTIN GOLDBERG, LEWIS W. BLUEMLE, JR. AND GEORGE D. WEBSTER, JR., Philadelphia, Pa. (introduced by J. Russell Elkinton).

Forty patients with acute renal failure were studied during oliguria and diuresis. In both periods, the urine flow was linearly related to excretion of sodium and total solute. The rate of increase of solute excretion during oliguria was generally constant and similar from patient to patient when plotted against a percentile time abscissa (days from onset of oliguria/duration of oliguria $\times$ 100). From this relationship a formula may be derived for predicting the approximate duration of oliguria in most patients. With few exceptions the peak of diuresis and the onset of rapid decline of blood urea nitrogen occurred simultaneously after a period of time following onset of diuresis equal to about one-half the duration of oliguria. During oliguria the ratio of urinary sodium to total solute varied from 0.03 to 0.35, the lowest values obtaining in severe trauma, burns, and congestive heart failure. The ratio C_{Na}/C_{Cr} varied considerably in individual patients. The ratio of non-electrolyte solute to total solute ranged from 0.16 to 0.67 and did not vary consistently with changes in composition of plasma solute occurring either spontaneously or during hemodialysis. With the advent of diuresis the urinary nonelectrolyte solute fraction was consistently 0.4 to 0.6. In several overhydrated patients the urine was hypotonic (U/P osmolality < 0.7) early in the oliguric phase. During oliguria, while urine flow is dependent on solute excretion, the lack of close correlation between changes in plasma and urine solute composition suggests that the tubules of a reduced number of nephrons are functioning in a qualitatively normal manner while glomerular filtration rate (GFR) is impaired. Diuresis is associated with an increasing urea osmotic load, an increasing number of participating nephrons and a rising GFR.

Specific Neural Stimulation of Norepinephrine Secretion by the Adrenal Medulla During Anoxia. ALAN GOLDFIEN, WILLIAM F. GANONG AND BERTRAM WISE, San Francisco, Calif. (introduced by Richard J. Havel).

We have previously reported that asphyxia (trachea clamp) and anoxia (100 per cent N_2) are unique stimuli to adrenal medullary secretion in the dog in that they regularly produce a proportionally greater increase in norepinephrine (N) than in epinephrine (E). Studies have been done to distinguish between interference with the conversion of N to E before release, and activation of a specific nervous pathway as the cause of this qualitative alteration in response. These experiments have been performed on dogs anesthetized with pentobarbital. Section of the splanchnic nerve or spinal cord and local or paravertebral procaine markedly depress the secretion of E and N. Infusion of cyanide into the arterial supply of the left adrenal in 5 dogs with bilateral adrenal cannulas caused an increase in the proportion of N on the left but not the right. However, the increase appeared late in contrast to the immediate change observed with intravenous cyanide or anoxia. In 4 dogs, the adrenal was perfused with oxygenated blood during asphyxia of the recipient. In these experiments the typical increase in N occurred. Infusion of anoxic blood into the adrenal of the normal dog did not increase N secretion. These results indicate that the N response results from nervous stimulation. Additional studies in 5 dogs in which chemoreceptor activity was modified by section of cranial nerves 9, 10 and 11 suggest that chemoreceptors may initiate the reflex increase in N since an increase in N secretion comparable with that observed in the normal dog was not found after this procedure.

Reactivity of the Renal Circulation in Essential Hypertension. ERVIN A. GOMBOS, WILLIAM H. HULET, PIERRE BOPP AND DAVID S. BALDWIN,* New York, N. Y.

The cause of arteriolar vasoconstriction in essential hypertension is unknown. Increased vascular reactivity has been proposed as a possible mechanism, and it has been suggested that sodium content of the vessel wall affects such responsiveness. In the present study, the reactivity of the systemic and renal circulations to infused noradrenaline and adrenaline has been compared, using percentile changes, in normotensive (N) and hypertensive (H) individuals on regular and restricted sodium intake. Noradrenaline caused equal increases in mean systemic pressure (Pm) and equal decreases in renal plasma flow (RPF) in N and H, producing comparable increases in renal resistance (RR) in N (+68 per cent) and H (+50 per cent). For given levels of Pm, RPF was lower in N than in H. Adrenaline did not change Pm but reduced RPF equally in N and H and to the same extent as did noradrenaline; RR increased less with adrenaline than with noradrenaline. Sodium restriction reduced Pm and RPF in H but did not affect either in N; Pm response to noradrenaline was exaggerated in H, unaffected in N; reductions in RPF were enhanced in N, unaltered in H. During sodium restriction, then, noradrenaline caused greater increases in RR in both H and N. Sodium restriction did not alter Pm response to adrenaline in either H or N, nor were the responses in RPF or RR affected in a consistent manner. We conclude: 1) Vascular reactivity to vasoconstrictor agents is not increased in either the systemic or renal circulation in essential hypertension. 2) Vascular reactivity is not decreased by sodium restriction in hypertension; indeed, systemic arteriolar reactivity is enhanced. 3) Autoregulation of the renal circulation in both H and N is suggested by our observation that greater increases in RR occur with noradrenaline, where Pm is increased, than with adrenaline, which does not affect Pm.

Potassium Excretion in Renal Disease. HARVEY C. GONICK, MORTON H. MAXWELL, RALPH E. CUTLER, J. THOMAS DOWLING AND CHARLES R. KLEEMAN,* Los Angeles, Calif.

A test was devised in an attempt[2] to quantitate the renal response to an acute K load at various levels of renal function. Following two 1-hour control periods, 0.75 mEq K per kg body weight was administered orally. Urine collections were made for three subsequent 2-hour periods. Bloods were drawn at the midpoint of each period. Samples were analyzed for K and creatinine. During the 6-hour test period, the increment in K excretion above the control was calculated. This was then expressed as a per cent of the administered load ("per cent excretion"). Twelve normal subjects and 16 patients with various types of renal disease were studied. The maximal increase in serum K was approximately 1 mEq per L in both groups. The renal disease patients differed from the normal controls in several important respects: 1) "Per cent excretion" of K was significantly lower, with no overlap (range, 0 to 16 per cent vs 24 to 71 per cent). When this figure was divided by glomerular filtration rate (GFR), some overlap between the two groups was present. A few patients, however, with minimal reductions in GFR, demonstrated a distinct abnormality in K excretion. 2) The elevation in plasma K tended to persist throughout the test period, whereas in the normal subjects the plasma K had returned to control values. 3) The increment in K excretion was invariably slower than in the normal group, in whom almost all of the increment occurred in the first 4 hours. 4) During the control periods, the ratio, K excreted/K filtered, was significantly higher than in the normal subjects (0.57 ± 0.85 vs 0.17 ± 0.08; $p < 0.05$). It appears that under ordinary circumstances the diseased kidney functions at near maximal capacity for K excretion and therefore responds inadequately when challenged with an acute K load. This test may serve as a useful index of tubular function in patients with minimal renal disease.

The Effect of Viral Infection on Cellular Protein Synthesis. IRVING GRAY, JOHN B. TASKER, JR. AND GEORGE R. FRENCH, Fort Detrick, Md. (introduced by William J. Darby).

The virus of Venezuelan equine encephalomyelitis (VEE), Trinidad strain, is a pantropic virus occurring in particularly high concentration for extended periods in the brain. Symptoms of this disease are typical of central nervous system derangement. Chemically, VEE is an RNA-containing virus, presenting the possibility that a mode of action of this microorganism could be through the alteration of cellular protein synthesis known to require cytoplasmic and microsomal RNA. Consequently, it was decided to study the effect of this virus on the protein synthetic ability of brain tissue. Male white mice, Bagg strain, were inoculated with approximately 400 LD_{50}'s of VEE; 50 animals were sacrificed each day for 6 days and the brains pooled in each group. Microsomes, soluble enzymes and RNA were prepared by differential centrifugation. Enzymes were incubated with microsomes in the presence of L-leucine-C^{14} and an ATP regenerating system. The reaction mixture was incubated in air at 37°C, with shaking, for 15 minutes. The microsomal protein was precipitated and uptake of radioleucine determined. The results of six complete assays are reported here. There is an increase in the incorporation of leucine-C^{14} into the microsomal protein, reaching a maximum on the third day following the challenge. There follows a sharp decrease to or below the control values. The increase occurs during a rising virus titer in the brain, while the fall occurs during the time when the virus titer in the brain has reached a maximum and remains relatively constant. The changes appear to be associated with the microsomal rather than the enzymatic fraction. Correlative histology and symptomatology will be discussed.

Experimental Human Trachoma in Six Blind Volunteers. J. THOMAS GRAYSTON,* SAN-PIN WANG, YEN-FEI YANG AND ROBERT L. WOOLRIDGE, Taipei, Taiwan, Formosa.

Six blind volunteers aged 11 to 24 who had no evidence of trachoma were infected in the eye with virus isolated from trachoma patients on Taiwan and grown in the yolk sac of embryonated eggs. Trachoma viruses have only recently been isolated and one purpose of this experiment was to show that the egg-grown virus was capable of reproducing trachoma disease in humans. The acute course of these infections has been reported in J. Amer. Med. Ass. The present report deals with the later clinical course of the infections, effects of a series of injections of a trachoma virus vaccine given to 3 volunteers beginning 2 months after infection, and responses to local and systemic therapy. Pannus, a typical sign of trachoma, developed in the one volunteer whose cornea was not damaged prior to the experiment. Conjunctival scars, diagnostic of trachoma, appeared in each infected volunteer between the seventh and ninth months after inoculation. Each of the 3 volunteers receiving placebo vaccine experienced a spontaneous spread of infection to his uninoculated eye. These spread infections, proven by virus isolation, occurred 1, 5 and 6 months after the original inoculation. Two volunteers who received trachoma virus vaccine experienced no spread of infection. The other one had been inoculated in both eyes. After 9 months all 6 were treated in both eyes with terramycin ointment 4 times per day for 1 month. Following therapy an acute relapse with active conjunctivitis in both eyes occurred in each of 3 volunteers who had received placebo. No recurrent signs of disease were seen in 3 volunteers who had received virus vaccine. Oral sulfa drug therapy was then given for 3 weeks and a complete cure resulted. It is concluded that trachoma was reproduced and that the vaccine probably exerted a favorable influence on the course of the disease.

Effects of Iodine on the Intermediary Metabolism of the Thyroid Gland and their Relation to Iodine-induced Inhibition of Thyroid Hormone Formation. WILLIAM L. GREEN AND SIDNEY H. INGBAR,* Boston, Mass.

Iodide maintains a paradoxical relation with the thyroid gland in that low concentrations are essential for hormonal synthesis, while high concentrations inhibit hormone formation transiently. Persistence of the latter effect is thought to produce iodide myxedema. Studies were performed to determine whether these effects of iodine could be mediated by an action on thyroidal intermediary metabolism, since previous studies indicated that TPNH formed during glucose catabolism is rate-regulating in hormone formation. Sheep thyroid slices were incubated in Krebs-Ringer phosphate medium in which iodide was isosmotically substituted for chloride. Metabolic effects of iodide were correlated with rates of hormonal synthesis determined from the percentile organification of I^{131} and the specific radioactivity of the medium. Iodide induced biphasic effects on hormone formation and glucose metabolism. Progressive increases in iodide concentration to approximately 10^{-4} M induced increasing organification of I^{127}. Concomitantly, there occurred a several-fold increase in the evolution of $C^{14}O_2$ from glucose-1-C^{14} and a less striking increase from glucose-U-C^{14} and glucose-6-C^{14}. Specific activity of evolved CO_2 increased considerably, while oxygen consumption, total CO_2 production, and glucose utilization were moderately increased. These stimulatory effects of iodide were abolished by methimazole and were not evident in liver, kidney, or adrenal. Equivalent concentrations of bromide had no apparent effect. At higher iodide concentrations (up to 5×10^{-2} M), hormonal synthesis declined and stimulatory effects on glucose metabolism were abolished or changed to inhibition. In thyroid homogenates, the increase in concentration of TPNH which followed addition of TPN was less in the presence of added iodide, especially the higher concentrations of iodide which decreased hormonal synthesis. These data indicate that thyroidal glucose metabolism and hormone formation are closely linked. They further suggest that the decrease in hormonal synthesis induced by iodide is related to an inhibition of those processes which generate TPNH.

An Intracellular Protein Intermediate for Hemoglobin Formation. WILLIAM B. GREENOUGH, III AND E. DONNALL THOMAS,* Cooperstown, N. Y.

Normal human and dog marrow were incubated *in vitro* with Fe^{59} and C^{14}-labeled amino acids. Ultracentrifuged lysates of the washed marrow cells were chromatographed on an IRC 50 column. A fraction moving with the solvent front in 0.01 M phosphate buffer at pH 7.0 (fraction I) contained an intermediate for hemoglobin formation. A delay of 5 to 8 minutes occurred before significant C^{14} or Fe^{59} appeared in the IRC 50 main hemoglobin fraction. Fraction I showed labeling within 1 minute and rose to plateau values in 15 to 30 minutes, remaining there until the rate of hemoglobin formation decreased at from 4 to 6 hours. When marrow cells incubated briefly with radioactive substrates were washed and reincubated in unlabeled substrate, a decrease in fraction I activity occurred, while the increase in hemoglobin activity continued. The turnover rate of Fe^{59} in fraction I accounted for all the Fe^{59} appearing in hemoglobin. In the presence of lead acetate, Fe^{59} entered fraction I but hemoglobin synthesis was blocked. Fe^{59} was also incorporated into hemoglobin by a lysate system of marrow with nuclei and stroma removed. In this system added unlabeled fraction I caused a fourfold enhancement of iron incorporated into hemoglobin, and a transfer of Fe^{59} from labeled fraction I to hemoglobin was demonstrated. Fraction I was heterogeneous; however, a single component containing nearly all the Fe^{59} could be demonstrated both by electrophoresis and by chromatography on DEAE cellulose. Heating to 80°C did not alter these properties but did remove much inactive material. The heat-stable component is a nonheme protein having 0.3 to 0.8 per cent iron stable to dialysis against acetate and EDTA at pH 4.6. Fraction I was clearly

distinct from $CdSO_4$-precipitated liver ferritin when compared electrophoretically and by DEAE chromatography. Similar N-terminal amino acid patterns, however, suggest a relationship to ferritin that is currently under investigation.

Studies on Pathogenesis of Typhoid Fever: Endotoxin and Vascular Reactivity. SHELDON E. GREISMAN, RICHARD B. HORNICK AND MERRILL J. SNYDER, Baltimore, Md. (introduced by Theodore E. Woodward).

Resemblances of clinical, pathological, and laboratory manifestations in typhoid fever to those induced by purified endotoxins suggest that the pathophysiologic alterations during *Salmonella typhosa* infection are mediated by endotoxin. This concept was supported in 1950 by demonstration of tolerance to the pyrogenic activity of endotoxin in subjects convalescing from typhoid fever. Such findings, however, lack confirmation and do not parallel observations following human infection with other endotoxin-containing bacteria. The present studies evaluate more critically the evidence for endotoxin activity during typhoid fever. Healthy young male volunteers were studied prior to infection. Responses of systemic arterial blood pressure and of nailfold capillary blood flow to intravenous infusions of norepinephrine were measured under standardized conditions. Subsequently, febrile responses following intravenous injection of 0.5 μg *Sal. typhosa* or 1.0 μg *Escherichia coli* were monitored over 8 hours. Viable *Sal. typhosa* (10^8 to 10^9) were administered orally, and illness was permitted to develop several days prior to chloramphenicol therapy. Febrile reactions to endotoxin and vascular responses to norepinephrine were assayed serially. Significant tolerance to pyrogenic activity of *Sal. typhosa* and *E. coli* endotoxin (fever indices below half the control levels) developed in convalescence, diminishing appreciably by the third week. Moreover, during typhoid fever, norepinephrine infusions at rates only one-half to one-quarter those of the control period induced comparable alterations in arterial pressure and capillary blood flow; fever per se was not responsible. Subjects not exhibiting overt illness did not develop endotoxin tolerance or increased reactivity to norepinephrine. Production of tolerance to pyrogenic activity of endotoxin and vascular hyperreactivity to catecholamines are documented paramters of endotoxin activity. The present findings support the thesis that *Sal. typhosa* endotoxin contributes significantly to the physiologic derangements in human typhoid fever. Resistance to development of overt illness following oral administration of viable *Sal. typhosa*, however, appears based upon mechanisms distinct from endotoxin tolerance.

Volume Regulation and Sodium Homeostasis Independent of Aldosterone. JACOB GROSSMAN, ARTHUR G. GOLDMAN AND MORRIS WOLFMAN, New York, N. Y. (introduced by Louis Leiter).

Vasopressin-induced water retention and increasing body fluid volume are normally accompanied by a natriuresis chronologically associated with a significant fall in urinary aldosterone excretion. Moreover, administration of relatively small amounts of sodium is followed by proportionate degrees of natriuresis, suggesting either that volume regulation is extremely sensitive or that there exists a mechanism for sodium homeostasis independent of fluid volume. An effort was made to separate these two influences and to ascertain their degree of dependence on aldosterone inhibition. Four subjects maintained on unrestricted (170 mEq per day) salt intake were given 9α-fluorocortisol (3 to 4 mg per day) during two separate 6-day periods. During one course of treatment, fluid intake was moderately severely restricted while during the other 6-day period, daily water intake, apart from that in food, was forced to 4 to 5 L. All diets and urinary collections were under metabolic control. The characteristic kaliuresis and sodium retention with progressive hypokalemia were observed in all cases. In 2 subjects, urinary sodium excretion was significantly less on the lower fluid intake despite the higher plasma sodium concentrations, suggesting that volume regulation predominated over that of sodium. In one subject natriuresis was approximately the same during both courses of 9α-fluorocortisol administration. While precedence of sodium homeostasis is suggested, the urine flow during high fluid intake was sufficiently enhanced to prevent a significant increase in volume. In one obese subject on a low (800) calorie diet, sodium excretion was greater during fluid restriction. The progressive increase in natriuresis during treatment indicates that such volume regulation may be independent of aldosterone level. Moreover, the data suggest that volume regulation underlies sodium homeostasis.

Effects of Single Amino Acids on Metabolism of Potassium and Hydrogen Ion. PHILIP W. HALL, III AND GEORGE J. GABUZDA,* Cleveland, Ohio.

The effects of single amino acids on acid-base balance and renal handling of electrolytes were investigated in 8 patients maintained on constant intakes of food and fluid. L-arginine (155 mM as free base) given orally daily for 3 days to each of 6 subjects resulted in increased urinary bicarbonate and potassium excretions, decreased hydrogen ion excretions (titratable acid and ammonia) and increases in urine pH. Blood electrolytes and pH were not altered. Similar changes occurred when L-lysine, but not when L-glutamic or L-aspartic acid was fed in equimolar quantities to some of the patients. Thus the effects noted occurred with basic (cationic) but not with acidic (anionic) amino acids. Subsequent studies were designed to determine whether the cationic amino acids exerted their effects primarily by influencing renal or extra-renal mechanisms. The responses of three patients to 100 mEq hydrochloric acid given orally daily for 3 days, with and without simultaneous L-arginine administration, were determined. When the acid load was given with L-arginine, there was 50 per

cent less reduction in serum bicarbonate concentration, and 40 per cent less hydrogen ion excreted in the urine than when hydrochloric acid was given alone. Urinary potassium excretions resulting from L-arginine given alone or with hydrochloric acid were increased and comparable. A slowly progressive acidosis was induced in a patient with chronic renal disease by administration of hydrochloric acid orally (25 mEq daily). Feeding L-arginine in addition to the acid resulted in stabilization of serum bicarbonate concentration and pH without change in hydrogen ion excretion. When L-arginine was discontinued, serum bicarbonate concentration promptly decreased further. These data are interpreted as follows: basic amino acids 1) behave as cations and probably displace some intracellular potassium, and 2) either decrease the production of hydrogen ions requiring excretion or increase total body buffering capacity.

Effects of P_{CO_2} and pH on the Plasma Binding, Turnover, and Tissue Uptake of Thyroid Hormones. MILTON W. HAMOLSKY* AND MYRON STEIN, Boston, Mass.

Knowledge is limited concerning the factors controlling the distribution of thyroid hormones between the specific binding proteins in plasma and the peripheral tissues. Our studies suggest an important determinant role of carbon dioxide tension (P_{CO_2}) and pH. The *in vitro* uptake by human erythrocytes of I^{131}-thyroxine (T4) and I^{131}-triiodothyronine (T3) from whole blood was strikingly increased in each of 24 euthyroid patients with hypercapnia, returning to normal when P_{CO_2} could be normalized. The *in vivo* turnover rate of infused I^{131}-T4 was similarly increased in 8 of these hypercapneic subjects. "Criss-cross" experiments revealed the alteration to be due to a plasma factor. Experimentally induced hypercapnia and hypocapnia resulted in a marked increase and decrease, respectively, in the *in vitro* erythrocyte uptake of T3 and T4. In dialysis studies, induced hypercapnia (pH stabilized by $NaHCO_3$ addition) caused increased dissociation of T4 and T3 from plasma proteins; conversely, hypocapnia resulted in increased plasma binding, decreased dialysis. A separate controlling role of pH was revealed in dialysis studies of T4 and T3 bound to 1) whole plasma, 2) highly purified inter-alpha globulin, 3) serum albumin, and 4) isolated prealbumin binding protein. Characteristic patterns over a wide pH range were observed for each binding protein, differing for each of the specific binding proteins, and differing for T4 vs T3. *In vivo* alteration of pH in dogs (temporary apnea, varying oxygenation, infusions of Tris or bicarbonate buffer) caused striking changes in T4 and T3 plasma binding—decreased below pH 7.4, increased above 7.4, with a sharp inflection point at 7.4. Comparable results have been observed in patients with altered acid-base balance. The possible physiologic significance of pH and P_{CO_2} changes in over-all thyroidal economy will be considered.

Clinical Applications of I^{125}. PAUL V. HARPER, KATHERINE A. LATHROP, HAROLD L. ENDLICH, WARREN SEIMENS AND ROBERT W. HARRISON, Chicago, Ill. (introduced by Leon O. Jacobson).

While the potential advantages of I^{125} for certain clinical and experimental studies have long been recognized, its high cost and low purity when prepared by cyclotron bombardment of tellurium have limited its use to animal material. Development of production methods using thermal neutron activation of xenon has made available large (0.5 c) amounts of relatively pure (>99 per cent) isotope. Briefly, its advantages as compared with I^{131} are: 1) The 60 day half-life provides long shelf-life and permits long-term metabolic studies; 2) the low energy dissipation rate greatly reduces radiation exposure of the patient in, e.g., thyroid scanning; 3) the low energy of the principal photon radiation (27.3 Kev) permits greatly reduced crystal size and shielding, and greatly enhanced detector and collimator efficiency. Even apart from these latter advantages, when the same microcurie dosage of I^{125} is used with unmodified equipment, it produces thyroid scans almost indistinguishable from those using I^{131}. In I^{125} Rose Bengal liver scans, metastatic nodules on accessible surfaces of the liver appear with markedly enhanced contrast because the radiation originating in the underlying liver is attenuated in passing through the "cold nodule." Since I^{125} may be counted in an ordinary well crystal with 60 to 70 per cent efficiency, studies involving small *in vitro* samples present no problem. It should be possible to use miniature shielded detector probes applied directly to the great vessels for cardiac output or shunting measurements during cardiac surgery, without undue interference from background radiation. There are, however, serious obstacles to the use of I^{125} where absorption in overlying tissue (HVL $\simeq$ 2 cm) is important, as in quantitative radioiodine uptake studies or brain scanning.

Recovery from Chronic Hypercapnia: The Critical Role of Chloride in Restoration of Normal Acid-base Equilibrium. GORDON D. HAYNIE, RICHARD M. HAYS, ADOLF POLAK AND WILLIAM B. SCHWARTZ,* Boston, Mass.

Balance studies have been carried out in eight dogs during recovery from chronic respiratory acidosis induced by a high CO_2 atmosphere. Four animals received a high salt diet and four others a low salt diet during the period of exposure to CO_2 and throughout the recovery period. At the end of the CO_2 period, plasma bicarbonate concentration ranged from 35 to 38 mEq per L in both groups; plasma chloride concentration was depressed. After being returned to room air, the high-salt dogs showed a prompt reduction in plasma bicarbonate concentration to a normal range of 21 to 24 mEq per L, and chloride concentration rose simultaneously to a normal level. By contrast, the low-salt dogs had a significantly smaller reduction in plasma bicarbonate

concentration, to values of approximately 27 to 30 mEq per L, and became mildly alkalotic; plasma chloride concentration showed little or no change. The fall in plasma bicarbonate in both groups usually took place without loss of bicarbonate in the urine. During the subsequent 6 days, the low-salt dogs stabilized their plasma bicarbonate levels in a range consistently above the normal; the plasma chloride concentration in these animals remained grossly subnormal. The persisent elevation of plasma bicarbonate concentration in the low-salt animals indicates that an elevated rate of hydrogen ion secretion persisted despite a normal plasma carbon dioxide tension. Potassium deficiency did not appear to be a factor in the difference observed between the low- and high-salt groups. When salt was added to the diet of the low-salt dogs, the plasma bicarbonate fell to normal and there was a reciprocal rise in plasma chloride concentration. The reduction in bicarbonate was achieved without urinary bicarbonate loss, but was associated with a marked suppression of acid excretion. Two additional studies of animals given a diet low in sodium but containing a moderate amount of chloride demonstrated that the acid-base disturbance and hypochloremia could be corrected solely by the provision of chloride. A possible mechanism to account for the observed effect of chloride deficiency in impeding the restoration of normal acid-base balance will be considered.

The Influence of Diet on the Excretion of Porphobilinogen in the Allylisopropylacetamide (AIA)-Treated Rat and the Porphyric Human. EMANUEL S. HELLMAN, DONALD P. TSCHUDY, JAMES A. ROSE AND ANNIE R. COLLINS, Bethesda, Md. (introduced by James A. Shannon).

The incidence of porphobilinogenuria induced in starved Sprague-Dawley rats by the injection or intragastric feeding of AIA could be decreased or abolished by progressively increasing the daily intake of carbohydrate (dextrin) or protein (casein hydrolysate) but not by increasing the intake of fat (cottonseed oil). In 3 humans with acute intermittent porphyria an inverse relationship was found between quantitative 24-hour urinary excretion of porphobilinogen and quantitative dietary intake of carbohydrate and protein. The activity of the enzyme that catalyzes the conversion of δ-aminolevulinic acid to porphobilinogen (δ-aminolevulinic acid dehydrase) per unit of liver nitrogen increases during starvation in the rabbit. Incubation of liver slices obtained from a starved rabbit with D-glucose or casein hydrolysate in phosphate buffer at pH 7.0 does not decrease the activity of δ-aminolevulinic acid dehydrase below that observed on incubation of slices in buffer alone. The effect of protein and carbohydrate on the quantitative 24-hour urinary excretion of porphobilinogen in experimental and human acute intermittent porphyria may be mediated through induced changes in the activity of δ-aminolevulinic acid dehydrase in the liver.

Transfer of Autoimmune Nephrosis in Rats by Means of Lymph Node Cells. E. V. HESS AND C. T. ASHWORTH, Dallas, Tex. (introduced by M. Ziff).

Recent studies by others have demonstrated that nephrosis may be induced in rats by the intraperitoneal injection of rat kidney homogenate with Freund's adjuvant. The disease is characterized by proteinuria, hypoproteinemia, hypercholesterolemia and hyperlipemia; histological studies have shown a membranous glomerulonephritis. The method of induction suggests an immunological basis for this condition. This possibility was investigated by attempting transfer of the disease to immunologically tolerant recipients by means of lymph node cells. A total of 44 rats was pretreated neonatally with spleen cells from the prospective donors. When lymph node cells from 12 nephrotic donors were injected intravenously into 17 prepared recipients, histological changes were observed in 12 of 14, major proteinuria in 11 of 14, and an increased serum cholesterol in 8 of 12 animals examined. In control experiments, no histological changes were noted in the kidneys of 11 prepared recipients who received cells for nonimmunized animals or animals injected with Freund's adjuvant alone. There were also no changes in the kidneys of a group of 16 animals consisting of nontolerant recipients of cells from nephrotic rats, recipients tolerant to rats other than the donors, and tolerant recipients who did not receive lymph node cells. Major proteinuria was not observed in any of the 27 animals of the control group and elevation in serum cholesterol occurred in only 2 animals. Immunofluorescent and electron microscopic studies have confirmed the histological observations. The former have shown the presence of γ-globulin in the involved glomeruli, and the latter have demonstrated swelling of the epithelial cytoplasm with obliteration of the foot processes and the presence of hyaline droplets in glomerular and tubular epithelium. The results of these experiments favor an immunological mechanism as the basis for the renal lesion produced in rats by injection of kidney tissue with Freund's adjuvant.

Studies in Man on Gonadotropin-responsive Adrenal Cortical Neoplasia. S. R. HILL, JR., HUGH DEMPSEY, W. R. STARNES, DITSIE PARTLOW AND W. B. NICKELL, Birmingham, Ala. (introduced by Tinsley R. Harrison).

The hormonal dependence and interrelationships of functioning endocrine neoplasia have not been elucidated fully as yet. The opportunity to investigate several aspects of this major medical problem was presented by a patient with a feminizing adrenal cortical tumor. Chromatographic separation and quantitation of individual urinary C_{18}, C_{19} and C_{21} steroids, and serial measurements of urinary total 17-ketosteroids (17-KS), 17-hydroxycorticosteroids (17-OHCS) and estrogens were performed before and after the infusion of corticotropin (ACTH), human chorionic gonadotropin (HCG), containing primarily LH-like activity and pregnant mares' serum gonadotropin (PMS), containing mainly

FSH-like activity, before and after removal of the tumor. There was no appreciable change in C_{19} or C_{10} steroids following ACTH. Elevated control levels of total estrogen (predominantly estriol) and 17-KS (predominantly dehydroepiandrosterone) were observed. Total estrogens doubled following HCG, and increased fivefold after PMS; both changes were due to an increase in the estrone fraction. Total 17-KS were unchanged following HCG, but appeared to increase after PMS. The data imply enzymatic transformations associated with tumor cells and are the first demonstration in man of a gonadotropin-responsive adrenal tumor. Such studies may lead to better understanding and more rational therapy of neoplastic disease.

On the Site of Viral Interference. MONTO HO, Pittsburgh, Pa. (introduced by Maxwell Finland).

The mechanism by which interferon, a mediator of interference and cell resistance, inhibits virus synthesis is unknown, but presumably it acts at an intracellular site. An approximation of this site was made by testing whether or not it blocks the infectivity of viral RNA. Infectious RNA was prepared from poliovirus type III by cold phenol extraction. Monolayers of about 10^6 chick embryo cells, which are genetically immune to the intact virus, were chosen as the substrate for RNA activity in order to restrict the production of intact virus to one cycle, and to eliminate the complicating interactions of intact virus on the host cell. An interferon (S-VIF) was obtained by the action of Sindbis virus on chick cells. Treated monolayers were overlaid with 0.25 ml S-VIF for 3 hours at 36° C and washed before inoculation. Titration of infectivity was by plaque formation on monkey kidney cells. Whereas control chick monolayers inoculated with about 100 plaque-forming units (p.f.u.) of infectious RNA produced from 10^3 to 7×10^3 p.f.u. intact poliovirus, treated cells simultaneously inoculated produced from 0 to 70 p.f.u. To determine whether this inhibition of RNA activity was explained by a difference in the adsorption or inactivation of RNA by cells treated with S-VIF, 1) residual RNA activity was measured after adsorption of RNA on treated and control cell layers, and 2) RNA was incubated with debris of treated and control cells. No difference was found. This inhibition could not be demonstrated when monkey kidney monolayers were substituted for those of chick cells, or if another interferon prepared by the action of a poliovirus type II on monkey kidney cells was used instead of S-VIF on chick cells. These studies together with available information suggest that the site of viral interference is at a point beyond the uncoupling of virus into its nucleic acid and protein components and that this action is mediated by soluble inhibitors that are cell-specific.

Correlation of Culture and Sediment Findings in Urinary Tract Infections. PAUL D. HOEPRICH, Salt Lake City, Utah (introduced by Maxwell M. Wintrobe).

Bacteruria $\geqslant 10^5$ per ml is diagnostic of infection in the genitourinary (GU) tract, but does not define the exact locus of such infection. Pyuria indicates inflammation in the GU tract, but does not reveal the precise site of such inflammation. While intensity of inflammation is reflected in degree of pyuria, only with nephritis does GU tract inflammatory exudate take on morphologically unique form as leukocyte casts (LC). Thus, coexistence of bacteruria and leukocyte casts should enable diagnosis of pyelonephritis. Evaluation of therapy should be aided by serial quantitative examinations of both urinary bacteria and sediment. Three hundred urine specimens from 72 patients were examined by conventional analysis, qualitative and quantitative culture, and enumeration of formed elements of the sediment after staining with: eosin-blue black ink-picric acid; crystal violet-safranin; benzidine-peroxide. Specimens were grouped as: *A*) bacteruria $\geqslant 10^5$ per ml with definite pyelonephritis, 42 specimens from 15 patients; *B*) bacteruria $\geqslant 10^5$ per ml without proved pyelonephritis, 60 specimens from 32 patients; *C*) bacteruria $< 10^4$ per ml with no history of recent antibacterial therapy, 37 specimens from 24 patients; *D*) the remaining 161 specimens were examined in the course of, or immediately following, antibacterial therapy. Pyuria, present in all specimens of *A* and *B*, was most marked in *A;* 84 per cent of *C* specimens had leukocytes, but fewer than *A* and *B* sediments. Pale staining ("viable") leukocytes were seen most often and in largest numbers with *A* and *B* specimens. LC were found in at least 1 specimen from each of the 15 patients of group *A*, in at least 1 specimen from 15 of the 32 patients of *B*, and in 5 group *C* specimens (2 chronic glomerulonephritis, 2 disseminated lupus, 1 acute renal failure). Hematuria was not a marked or frequent finding. Treatment which effected disappearance of bacteruria and LC without cure of pyelonephritis usually did not eliminate pyuria. Both diagnosis and therapy of GU infections are aided by quantitative and qualitative culture and sediment examination.

Renal Sodium Excretion in Man Following Small Increments of Intravascular or Total Extracellular Volume. WALTER HOLLANDER, JR.,* Chapel Hill, N. C.

Much circumstantial evidence suggests that renal sodium excretion is regulated by changes in some component of intravascular volume, and alterations of intravascular volume affect aldosterone excretion. Nonetheless, the question of whether the intravascular or interstitial compartment mediates momentary changes in renal sodium excretion remains unsettled and debated. The current study was undertaken to reexamine this problem utilizing a design suggested by studies of Strauss and co-workers. Subjects were healthy young men with very low basal rates of sodium excretion, having been on a sodium-restricted intake for 1 week. All studies were conducted during the afternoon by the same person in an unvarying manner. A 500 ml positive water balance was maintained throughout. Electrolyte excretion was measured during 30-minute intervals for 2 hours before and 2.5

hours after a 25-minute infusion of either 150 ml of an approximately isoncotic solution of albumin in slightly hypotonic saline (5 studies) or an equal volume of control solution without albumin but otherwise identical to the experimental solution including the albumin vehicle (5 studies). Sodium excretion increased progressively following infusions, probably more after albumin than after control solution (p just less than 0.05). This agrees with studies of Strauss and co-workers in suggesting a highly sensitive "volume receptor" regulating sodium excretion, and it favors an intravascular location. Three additional studies using only 25 ml of control solution and three using a mock design (subjects thought they received an infusion which actually was diverted so as not to enter the vein) revealed increments of sodium excretion comparable to those following 150 ml of control solution; hence, the latter rise, and part of that observed following albumin, presumably resulted from factors other than extracellular expansion. Although not identified in these experiments, such factors obviously represent an important hazard to interpretation of this type of study.

Myocardial Blood Flow as Indicated by the Disappearance of NaI^{131} from the Heart Muscle. WILLIAM HOLLANDER, IRVING M. MADOFF AND ARAM V. CHOBANIAN, Boston, Mass. (introduced by Robert W. Wilkins).

Previous studies have indicated that the removal of an ion injected into a tissue is a function of blood flow. In the present work, the rate of disappearance of NaI^{131} (0.5 ml) injected percutaneously or during open thoracotomy into the heart muscle of dogs and of human subjects was studied as an index of myocardial blood flow. NaI^{131} activity, as determined by external monitoring of the injection site, decreased at an exponential rate with a mean $T_{\frac{1}{2}}$ of 1.5 minutes. In anesthetized dogs the disappearance of NaI^{131} slowed as the coronary artery was narrowed with a clamp and stopped completely following total occlusion of the artery. Following the release of the clamp (and occurrence of reactive hyperemia) the disappearance of NaI^{131} accelerated markedly. In patients with coronary artery disease the exponential removal of NaI^{131} was markedly impaired, being $\frac{1}{4}$ to $\frac{1}{15}$ as rapid as that in "normal" subjects. Sublingual nitroglycerine (0.8 mg) reduced blood pressure but did not increase the rate of removal of NaI^{131} from the myocardium. Exercise in the same patients increased the removal of the isotope without a proportionate increase in blood pressure. Internal "phlebotomy" (by cuffing), when accompanied by hypotension, reduced the removal of NaI^{131}. It is concluded that the disappearance of NaI^{131} from the heart muscle appears to be an index of myocardial blood flow. In coronary artery disease, coronary flow is impaired but appears capable of increasing. The concomitant reduction of blood pressure may account for the failure of nitroglycerin to augment flow.

Mechanism of Endotoxin-induced Coagulation Acceleration. HERBERT I. HOROWITZ, ROGER M. DES PREZ AND EDWARD W. HOOK, New York, N. Y. (introduced by Paul Reznikoff).

A shortening of the clotting time of whole blood or of platelet-rich plasma in the presence of endotoxins of gram-negative bacteria has been reported by McKay and by Robbins and Stetson. The present investigation was undertaken to study the mechanism of endotoxin-induced acceleration of coagulation. The formation of blood thromboplastin depends on an interaction of platelet phospholipid and product I. The phospholipid of intact platelets is not readily available to participate in thromboplastin formation. However, if the platelets are altered, as by thermal injury, phospholipid is more available and an acceleration of clotting can be demonstrated. The availability of platelet phospholipid to participate in coagulation is determined by measuring the clotting time of recalcified platelet-rich plasma after addition of an excess of product I ("product I substrate time"). The product I substrate time of plasma with intact platelets is greater than 20 seconds. In contrast, when platelet phospholipid is fully available the product I substrate time is less than 10 seconds. Incubation of platelet-rich plasma with *Escherichia coli* endotoxin produces a rapid progressive decrease in product I substrate clotting time as compared with controls incubated with saline. The increased availability of phospholipid as determined by this method is apparent by 15 minutes' incubation at 37° C and with optimal dose is maximal by 60 minutes. The release of phospholipid from platelets incubated with endotoxin is accompanied by platelet aggregation and fusion ("viscous metamorphosis"), and roughly parallels transfer of serotonin and a bactericidin for *Bacillus subtilis* from platelets to plasma. Changes in individual clotting activities other than platelet phospholipid have not been found. The present *in vitro* studies utilizing platelet-rich plasma from rabbits suggest that endotoxins increase the availability of phospholipid for the clotting process by an action on platelets.

Mechanism of Action of Thrombin on Platelets. DUDLEY P. JACKSON,* HANS J. SCHMID AND J. RICHARD GAINTNER, Baltimore, Md.

Thrombin, a proteolytic enzyme, initiates certain reactions of platelets including aggregation, clot retraction and release of serotonin. Fibrinogen, a known substrate of thrombin, was investigated for its possible role in these reactions. Concentrates of fresh, washed, human platelets were frozen and thawed repeatedly. The supernatant fluid clotted upon addition of thrombin, indicating the presence of "platelet fibrinogen" as described by others. Fibrinogen could not be obtained from platelets which previously had been incubated with trypsin, although trypsinized platelets appeared morphologically intact. Fibrinogen, therefore, appears to be present on the surface of platelets and can be removed without destruction of the cells. Fresh human platelets suspended in

serum were grossly aggregated by addition of thrombin. Platelets treated with trypsin and subsequently incubated with serum and thrombin did not show significant aggregation. Platelets treated with trypsin and resuspended in purified bovine fibrinogen in a buffered solution of glucose induced retraction of clots formed after addition of thrombin. Platelets incubated with serum and thrombin remained intact until the addition of fibrinogen permitted the occurrence of clot formation and retraction and the irreversible changes of platelets associated with these phenomena. These results suggest that fibrinogen is the substrate of thrombin in the production of platelet aggregation and in the initiation of clot retraction. Platelets treated with trypsin or thrombin released more than 70 per cent of their serotonin. Platelets so treated retained their ability to take up serotonin again. Thrombin blocked the uptake of serotonin by trypsinized platelets, suggesting that fibrinogen is not involved in this reaction.

Reovirus Type I as an Etiologic Agent of the Common Cold. GEORGE GEE JACKSON,* ROBERT L. MULDOON AND GLORIA S. COOPER, Chicago, Ill.

Reovirus, prototype I (ECHO 10) was first recognized in 1954 when it was recovered from the stool of a healthy subject. Later, antigenically related types 2 and 3 were isolated from the respiratory tract as the presumptive cause of acute coryzal illness. We have isolated Reovirus type I from a nasal secretion collected in 1954, from a subject with a common cold. The strain was retained by a 50 mμ filter, ether-resistant, hemagglutinated human group O erythrocytes using a neuraminic acid receptor, and had negligible neuraminidase activity. The filtered nasal secretion was infectious in volunteers and caused a cold-like illness in 37 per cent of 185 subjects after the initial challenge and in 2 of 11 upon rechallenge. The monkey kidney tissue culture harvest was given to volunteers and no illness was produced. However, among 8 subjects given a dose of 10,000 TCD_{50}, all showed a response in neutralizing antibody. Volunteers who received the nasal secretion showed no relationship between the initial level of neutralizing antibody against Reovirus type I and the development of clinical illness. Following the challenge, 9 of 20 subjects who developed a clinical infection and 6 of 18 volunteers who did not, showed an antibody rise to Reovirus type I. Among volunteers given other infectious nasal secretions and who developed respiratory illness a rise was seen in only 1 of 7 tests involving 35 subjects. The data clearly establish the presence of Reovirus type I in the respiratory secretion. Volunteers were infected but there is much doubt as to the etiologic role of reovirus in the causation of respiratory illness. The tissue culture virus infected the nasal mucosa without producing symptoms. It seems likely that the clinical infections produced by the common cold secretion were caused by some other undetected virus.

N^{15}-Glycine Labeling of Stercobilin in Refractory Anemia. G. WATSON JAMES, III* AND LYNN D. ABBOTT, JR., Richmond, Va.

Stercobilin excretion has been reported as being elevated without a concomitant accelerated peripheral erythrocyte destruction in dyserythropoiesis (James and Abbott). Three additional patients with refractory normocytic or macrocytic anemia, hyperplastic bone marrow, and leukopenia, but without thrombocytopenia, have now been studied. Two 500-mg oral doses of N^{15}-glycine (31 atom per cent excess N^{15}) were given at a 4-hour interval. Stercobilin was crystallized from stools collected from five 72- to 96-hour periods. Venous blood was taken at 96-hour intervals for hemin crystallization. N^{15} analyses were performed in a Neir-type mass spectrometer. Serial hematologic studies, serum bilirubin and quantitative fecal urobilinogen were performed. Two normal control subjects showed N^{15}-stercobilin concentrations of 0.022 and 0.021 atom per cent excess N^{15} in the first period, with rapid decline to "normal" (0.013, 0.012) during the next 96 hours. The study patients had N^{15}-stercobilin concentrations of 0.065, 0.058, and 0.023 in the first period. These increased to 0.100, 0.120, and 0.074 atom per cent excess N^{15} in the second 96-hour period and "normal" values were not reached in the 20 days of study. Hemin-N^{15} in the control patients was 0.018 and 0.014. Two study patients had 0.020 maximum hemin N^{15} concentration and the other had a hemin-N^{15} label of 0.044. Active peripheral hemolysis and shortened erythrocyte life span were not evident during the study period. One of the three patients eventually developed acute granulocytic leukemia, and one of the previously reported patients died with this disorder. These data of stercobilin excretion are similar to those previously described and to those observed in pernicious anemia and folic acid deficiency. The data support our previous hypothesis that anabolic stercobilin, reflecting bile pigment synthesis, is markedly stimulated in refractory anemia.

Isolation of Inclusion Conjunctivitis Virus in the United States. ERNEST JAWETZ,* LAVELLE HANNA, PHILLIPS THYGESON, CARL MORDHORST AND CHANDLER DAWSON, San Francisco, Calif.

Inclusion conjunctivitis is an acute nonbacterial eye disease most commonly seen in newborn children, occasionally in adults. It begins with redness, edema, and infiltration of the conjunctiva and a mucopurulent exudate. It does not involve the cornea and, in contrast to trachoma, all lesions heal spontaneously in weeks or months. In epithelial cells from infected conjunctiva inclusion bodies are seen microscopically which closely resemble those of trachoma. Similar inclusions occur sometimes in epithelial cells from the mother's cervix and it is evident that the newborn acquires the infection from the mother's genital tract. From three infants with typical acute inclusion conjunctivitis, viruses were isolated for the first time in America by yolk sac inoculation

of embryonated eggs. Sixteen similar patients yielded no virus. Four to 5 blind passages were required until the strains were lethal for eggs and showed abundant virus microscopically in yolk sac smears. In morphology, stability, growth in eggs, and susceptibility to antimicrobial drugs, these agents resembled trachoma viruses isolated in this laboratory. Inclusion conjunctivitis viruses reached titers of 10^6 to 10^7 egg LD_{50} per ml. About 20 egg LD_{50} constituted a minimal infective dose for cynomolgus monkey eyes, resulting in clinical disease similar to that of man and in the development of inclusion bodies. From experimental infection of the monkey eye, virus was re-isolated for at least 3 weeks. We are searching for laboratory characteristics which would differentiate the viruses of trachoma and inclusion conjunctivitis, to parallel the evident difference in pathogenicity for man.

The Metabolism of Tritium-labeled Folic Acid in Man. D. G. JOHNS AND A. S. V. BURGEN, Montreal, Canada (introduced by Douglas G. Cameron).

After intravenous injection of 15 μg per kg of tritium-labeled folic acid, the plasma concentration falls rapidly so that after 2 hours only 3 to 4 per cent of the dose remains in the plasma and extracellular space. Since only 20 per cent of the dose had been excreted in the urine by this time, the remainder had entered the intracellular space. With smaller doses the excretion is smaller and the rate of tissue uptake accelerated. If 30 mg of unlabeled folic acid is injected 1 or 2 hours after the tritiated material it will displace radioactivity from the cells and raise the blood level of radioactive folic acid. Most of the injected radioactivity is then excreted in the urine in the succeeding few hours; 60 to 80 per cent is recovered as apparently unchanged folic acid, the remainder is due to folinic acid, *p*-aminobenzoylglutamate, a pteridine and two unidentified compounds. Folic acid is evidently stored in the cells largely unchanged. However if there is a delay of 72 hours before injecting the nonradioactive folic acid, much less of the dose is recovered, indicating sequestration of slow metabolic transformation of the stored folic acid. The rate of uptake of folic acid into the cells can be depressed by amethopterin.

A Study of Respiratory Syncytial Virus Infection in Adult Human Volunteers. K. M. JOHNSON, R. M. CHANOCK,* H. M. KRAVETZ, D. RIFKIND AND V. KNIGHT,* Bethesda, Md.

Recent studies have shown that respiratory syncytial virus (RS) is associated with acute respiratory disease in children. Illnesses in children were often characterized by fever and signs of pulmonary involvement including pnuemonia and bronchiolitis. Vouunteer studies were undertaken to determine pathogenic properties of this agent in adults. Forty-one men were given RS virus intranasally. Thirty-three individuals were successfully infected, and 20 developed respiratory illnesses. None of the 20 control volunteers showed evidence of RS infection. Illnesses were mild, afebrile and consistent with descriptions of the "common cold." The observed association between virus challenge and subsequent illness was strengthened by the finding that illness never preceded initial RS virus isolation and the fact that illness occurred for the most part in individuals who shed detectable virus for an interval of more than 2 days. Initial virus isolation from volunteers subsequently developing illness generally occurred on the third or fourth postinoculation day. Circumstances of several initial isolations made after the fifth postinoculation day suggested that these might have represented secondary infection. Neutralizing and complement-fixing antibody responses to RS virus occurred predominantly in subjects who developed clinical illness. This finding together with the correlation of illness with duration of virus shedding suggested that the induced respiratory disease reflected the extent of RS viral infection. RS virus infection in these volunteers represented reinfection, since all men had detectable RS neutralizing antibody prior to challenge. Pre-existing levels of antibody, however, did not influence subsequent RS infection or susceptibility to respiratory illness.

Increased Turnover of Phosphoribosylpyrophosphate, a Purine Nucleotide Precursor, in Certain Gouty Subjects. O. W. JONES, D. M. ASHTON AND J. B. WYNGAARDEN,* Durham, N. C.

This study in gouty subjects was designed to evaluate the turnover of phosphoribosylpyrophosphate (PRPP), a compound intimately involved in the primary regulatory mechanism controlling purine synthesis *de novo*. The reaction in which PRPP and glutamine form phosphoribosylamine (PRA) is the site of feedback control mediated by ATP. A defect of this control might be reflected in a disturbance of PRPP turnover. PRPP is also involved in other reactions, one of which, conversion of imidazoleacetic acid (IAA) to its ribonucleotide, is the basis of this study. Glucose-C^{14} was administered orally to label the ribose moiety of PRPP, and IAA orally to permit sampling of PRPP by analysis of urinary IAA-riboside (IAAR). In 4 control subjects 0.01 to 0.06 per cent of C^{14} appeared as ribose-C^{14} in IARR in 10 hours. (Hiatt found 0.05 to 0.06 per cent in 3 controls.) In 3 gouty subjects who excreted urate excessively, 0.16, 0.20, and 0.31 per cent of C^{14} appeared as IAAR. Time courses of specific activities showed early maxima, three times greater than normal. Relative specific activity (RSA) of ribose ranged from 1.4 to 4.8 in 7 controls, and was 10.9, 14.0, and 16.6 in gouty subjects. C^{14} incorporation and RSA values correlated with urinary urate excretion. In contrast, conversion of IAA to IAAR was not increased in these subjects. One additional gouty subject with normal 24-hour urate excretion, low urate/inulin clearance ratios, and normal glycine-1-C^{14} incorporation values on two occasions, showed normal glucose-C^{14} incorporation into IAAR, and normal RSA values. These results suggest *1*) that gouty subjects who

clearly overproduce urate may have an ineffective feedback control at the PRPP to PRA step, and *2*) that gouty subjects with normal urate outputs may, at least in some cases, have hyperuricemia on the basis of some other mechanism.

Acute Defibrination Syndrome in Metastatic Carcinoma: Role of Tumor Emboli. MUSTAFA KARACA, MARIO STEFANINI* AND BASIL KEKIS, Boston, Mass.

A 72 year old male with bronchiogenic carcinoma developed petechiae and ecchymoses and later died of uncontrollable hematuria. Early in the disease, laboratory studies showed: fibrinogen 61 mg% [tyrosine and $(NH_4)_2SO_4$ precipitation method]; platelet count, 76,000 per mm^3; bleeding time, over 10 minutes; tourniquet test, positive; concentration of AHG, prothrombin and factor V, 33, 60 and 20 per cent, respectively. The "serum factors" were unaffected. No general anticoagulants, antithromboplastin, antithrombin III and VI could be demonstrated. Esterolytic and fibrinolytic activity of plasma was normal; activity of proactivator, activator, plasminogen and antiplasmin was also normal. Survival of fibrinogen and platelets, studied by radioactive and by transfusions technics, showed a much shortened life span. All results remained unchanged in the course of the disease. Autopsy findings showed massive infiltration of lungs, adrenals and liver by tumor tissue. No material stained by PAS or for collagen was seen in tissues or deposited on the intimal wall of organs. *In vitro,* tumor as well as normal tissue showed no proactivator or activator activity. Fresh and acetone-dried normal and tumor tissue showed comparable thromboplastic activity. Numerous tumor emboli were seen within the lung vessels, surrounded by thrombi, composed of fibrin and presumably platelets. Defibrination syndrome of disseminated tumors has been attributed to many mechanisms: failure of fibrinogen production, fibrinolysis, intravascular deposition of fibrin on the intimal wall complicated by fibrinolysis and depolymerization of fibrinogen by proteolysis. The present case demonstrates an additional mechanism—namely, the role of tumor emboli in causing massive intravascular defibrination in a fashion similar to that of amniotic squamae during accidents of pregnancy.

Relationship of Bacteriuria to Hypertension: An Epidemiological Study. EDWARD H. KASS,* WILLIAM E. MIALL AND KENNETH L. STUART, Boston, Mass, Penarth, Wales, and Jamaica, West Indies.

The role of pyelonephritis in the pathogenesis of hypertension is unclear. Asymptomatic bacteriuria has been linked to pyelonephritis and occurs frequently in hospital populations, but its prevalence in nonhospital populations is unknown. The prevalence of bacteriuria and its relationship to blood pressure were investigated in defined rural and urban populations in Jamaica, and in defined mining and agricultural populations in Wales. Over 95 per cent cooperation within the selected populations was obtained. Voided urines for bacterial counts (repeated as needed to establish 96 per cent confidence limits at the level of 10^5 colonies per ml), blood pressures by standardized technique, and brief medical history were obtained. In 3,057 females, the prevalence of bacteriuria was 4.4 per cent (3.4 to 7 per cent in the different groups), with slightly higher prevalence in Wales than in Jamaica. In males, the prevalence was < 0.5 per cent. Bacteriuric females had significantly higher blood pressures than did nonbacteriurics. The prevalence of bacteriuria in females with diastolic pressures > 90 mm was 7.1 per cent and with diastolics < 90 mm was 3.4 per cent ($p < 0.01$). Bacteriuria was more prevalent among married than single women, rose with age and parity, was not familial in occurrence, was not related to diabetes mellitus or to glycosuria, and was not related to albuminuria. Bacteriurics were separable from nonbacteriurics on several grounds, such as the differences in sex incidence of bacteriuria, and the finding that in bacteriurics increased blood pressures are related to increased parity whereas in nonbacteriurics there is an inverse relationship between parity and the height of the blood pressures. The data favor the hypothesis that bacteriuria is etiologically related to hypertension rather than that it is secondary to hypertension and suggest preventive possibilities. Population studies of hypertension are subject to error unless asymptomatic bacteriuria is considered.

Iron and Protein Kinetics Studied by Means of Doubly-labeled Crystalline Transferrin. JAY H. KATZ, Boston, Mass. (introduced by Charles P. Emerson).

Crystalline transferrin (Tr) was labeled with I^{131} to yield a product which contained 0.5 to 0.8 equivalents of iodine per molecule of protein. Yields were 90 to 95 per cent and chromatographic analysis indicated that the iodine was incorporated chiefly as monoiodotyrosine. The iron binding capacity and other physical and immunologic properties appeared to be unaltered. Behavior with reticulocytes in an *in vitro* system was similar to that of uniodinated Tr. Iodinated Tr was administered intravenously to 7 normal adults as the Fe^{59} complex, the level of saturation varying from 35 to 100 per cent. The disappearance of both tracers from the plasma was followed for the first 3 hours and daily thereafter for 17 to 23 days. The clearance and RBC utilization of iron were similar to that found using whole plasma, although the clearance appeared to be somewhat more rapid in the 3 subjects given 100 per cent saturated Tr. Initial diffusion of Tr from the vascular space did not exceed 7 per cent per hour, roughly paralleling albumin in this regard. Hence, if Tr is bound on surface sites of erythropoietic cells, as was suggested by *in vitro* studies, such binding must be of brief duration and, having yielded its Fe, the Tr must be released directly back into the vascular space. Equilibration with the extravascular compartment was achieved in 4 to 5 days. The total exchangeable pool was 0.23 to 0.30 g per kg, 55 to 62 per cent of which was extravascular. After equilibration the Tr disappeared at a single exponential

rate, with a half-life of 6.7 to 8.4 days. The results indicate Tr lightly labeled with iodine maintains its integrity as a transport protein, has a reproducible half-life in normal subjects, and should be of value in the study of aberrations in iron and transferrin metabolism observed in many disease states.

The Production of Fever in Rabbits with Extracts of Tissue Culture Cells Infected with Coxsackie Virus. M. KENTON KING, St. Louis, Mo. (introduced by Carl G. Harford).

Extracts of polymorphonuclear leukocytes have produced fever in animals following intravenous injection. Influenza viruses contained in amniotic fluid from infected embryonated eggs are also pyrogenic. However, viruses grown in tissue culture which is free of the products of inflammation present in the infected chick embryo have not previously produced fever. The following experiments describe the characteristics of fever produced in rabbits by Coxsackie virus grown in monkey kidney cells, a system in which leukocytic pyrogen is excluded. Bottles containing monolayers of monkey kidney cells grown in nutrient media were inoculated with Coxsackie B_1 virus. When the cytopathic effect became marked the cells were collected, pooled, and washed free of nutrient media. Subsequently the cells were suspended in 0.85 per cent saline and disrupted by alternate freezing and thawing before testing. Monkey kidney cells, either whole or disrupted, which had not been inoculated with virus failed to produce fever following injection into rabbits. Consistent fevers were produced, however, by aliquots of disrupted cells containing Coxsackie B_1 virus. The fever regularly followed a latent period of 45 minutes and produced two peaks, one at 90 minutes and the second at 3 to 4 hours. Identical fevers were produced in rabbits previously made tolerant to the fever-producing effect of bacterial endotoxin. Rabbits injected on consecutive days with Coxsackie virus pyrogen exhibited tolerance by Day 3. The pyrogenic activity was not diminished even though aliquots were heated to 70° C for 20 minutes, indicating that the fever is not due to active infection. These findings indicate that extracts of monkey kidney cells infected with Coxsackie virus in the absence of leukocytes produce a characteristic fever following intravenous injection into rabbits. Attempts to obtain similar results with adenovirus and vaccinia virus grown in HeLa cells were not successful.

Studies of Intracellular Amino Acid Transport in Man as an Index of Protein Metabolism: Effect of Growth Hormone. DAVID M. KIPNIS,* MONROE GROSS AND WILLIAM H. DAUGHADAY,* St. Louis, Mo.

Protein synthesis and intracellular amino acid transport are so coordinated in intact cellular systems that changes of rate in one are mirrored by similar changes in the other. A technique has been developed for examining amino acid transport in man using the nonmetabolizable amino acid, α-aminoisobutyric acid (AIB). The results in 9 normal subjects (5 male, 4 female) and in 2 hypophysectomized patients before and after receiving human growth hormone (HGH) form the basis of this report. AIB-1-C^{14} (94 μg per kg body weight, sp. act., 1.6 mc per mmole) was injected intravenously in the nonfasting state. Blood levels and urine recoveries were recorded for 5 to 10 days. AIB disappearance from blood followed a multi-exponential function representing initial distribution in extracellular space, transport into intracellular water and renal clearance. AIB distribution between intracellular water and plasma reached equilibrium after 10 to 12 hours, remaining relatively constant thereafter. AIB was rapidly excreted; 50 per cent of the administered dose appeared in urine in 0.3 to 0.7 day in females and in 1.0 to 1.5 days in males. Over 98 per cent of the total dose was recovered in urine. Renal clearance was 60 to 75 ml per minute in normal and 40 to 50 ml per minute in hypophysectomized subjects, the latter rate increasing to normal levels, as did creatinine clearance, after HGH treatment. The apparent volume of distribution of AIB (V_{AIB}), normalized to 70 kg body weight, was 220 to 280 L in males and 134 to 157 L in females; indicating marked intracellular concentration. In two hypophysectomized patients, HGH treatment increased the low control V_{AIB} levels from 67 to 123 L to 138 and 331 L, respectively, and resulted in marked nitrogen retention. This technique affords a rapid and sensitive measure of growth hormone action and may be applicable for assessing the effects of other hormones and dietary factors on protein metabolism.

Studies on Factors Which May Contribute to the Pathogenesis of Staphylococcal Infection. M. GLENN KOENIG AND DAVID E. ROGERS,* Nashville, Tenn.

The factors which render certain staphylococci capable of producing infection are not clearly understood. While considerable information is available regarding the microbial factors which determine virulence of other gram-positive cocci, the features which characterize "pathogenicity" among strains of staphylococci have not been defined. A single strain of staphylococcus has been shown to form two distinct colonial variants which can be readily identified in special media. These two variants can be derived from a single clone. While both variants produce pigment and coagulase, only one variant consistently produces fatal intraperitoneal disease in mice. The other variant is promptly eradicated *in vivo*. Studies of these variants and the experimental infection they produce have brought to light factors which may act to initiate progressive staphylococcal disease. While both strains are able to grow in the presence of serum, only the virulent variant possesses components which cause it to resist intraperitoneal phagocytosis. These components are antigenic and may be located at the cell surface. Immunization with vaccines prepared from the virulent variant promote intraperitoneal phagocytosis and protect against fatal disease. Vaccines prepared from the avirulent variant do not. The behavior of these two staphylococcal variants, the relationship of certain biologic properties to virulence, and the charac-

teristics of staphylococcal-host cell interactions in an experimental mouse infection will be considered.

Study of "Basket Cell" Metabolic Activity in Human Leukemia by Tritiated Thymidine Radioautography. DONALD R. KORST, EUGENE P. FRENKEL AND JOAN E. WILHELM, Ann Arbor, Mich. (introduced by Sibley W. Hoobler).

The term "basket cell," also known as smudge cell, degenerating cell, naked nuclei, fragmented cell, ghost cell, or nuclear shadows of Gumprecht, covers a group of cell fragments seen in blood and bone marrow. Their origin is generally thought to be from mechanical pressure in the preparation of smears or films. Recent studies with radioautographs utilizing tritiated thymidine (H^3T) reveal that many basket cell forms take up the isotopically-labeled material in high concentration and are perhaps part of an active metabolic pool or primitive proliferating pool of Cronkite. The marrow and peripheral blood from 12 patients with acute and chronic leukemia have been studied *in vivo* and *in vitro* with H^3T. Patients studied *in vivo* received 200 μc H^3T. (specific activity 5.2 mc per mmole), and blood was obtained serially from 15 minutes to 72 hours. Bone marrow was obtained by aspiration at 48 or 72 hours. Five μc H^3T was added *in vitro* to 3-ml aliquots of blood and incubated 5 minutes to 2 hours prior to preparing radioautographs. Slides were then dipped in methyl alcohol to fix and remove thymidine not incorporated into DNA, eliminating a possible artifact. High resolution radioautographs were prepared with Kodak dipping emulsion and stained with Wright's stain. The basket cell concentrates H^3T within 15 to 30 minutes following *in vitro* introduction of the isotope. The *in vivo* label was recognized within 2 hours. This cell is different from the usual fragmented granulocyte or lymphocyte and occurs primarily in leukemia or lymphomas. Mechanical injury to cells *in vitro* does not produce basket cells that incorporate H^3T. It is proposed that this fragmented or disintegrating cell retains some DNA-synthesizing ability and may be a reflection of general proliferative activity. The concentration of H^3T in these cells appears early in the marrow and blood indicating active DNA synthesis. The basket cell is a metabolically active cell or cell fragment in contrast to denegerating granulocytes and lymphocytes. This cell is morphologically identifiable and perhaps is another means of determining proliferative activity in leukemia and lymphoma.

Evidence for Deficiency of Vitamin B_6 in Non-tropical Sprue. O. DHODANAND KOWLESSAR, LORRAINE J. HAEFFNER, GORDON BENSON AND MARVIN H. SLEISENGER,* New York, N. Y.

The urinary metabolites of tryptophan—5-hydroxyindoleacetic acid (5OHIAA), indole-3-acetic acid (IAA), kynurenine (Kyn), xanthurenic acid (XA), and indican—were studied in 10 normal subjects and in 21 patients with non-tropical sprue; 5 received no therapy and 16 were treated with a gluten-free diet. All of the latter were in remission, but only 3 had normal fat absorption. Measurements were made on an 8-hour collection of urine for the following periods: control; after loading with 4.0 g L-tryptophan; after 90 mg. pyridoxine (vitamin B_6) intramuscularly; and after reloading with 4.0 g L-tryptophan plus 90 mg pyridoxine. Reported results (mg) are means and standard deviations; normal values before and after 4.0 g L-tryptophan are: 5OHIAA, 1.6 ± 0.6 and 3.4 ± 1.2; IAA, 3.6 ± 3.0 and 21.8 ± 11.8; Kyn, 0.4 ± 0.15 and 29.2 ± 18.4; XA, 5.4 ± 3.3 and 10.3 ± 3.9; indican, 30.3 ± 16 and 40.6 ± 14.3, respectively. In 5 untreated patients the excretion of 5OHIAA, IAA, Kyn, XA and indican was increased (3.2 ± 0.6; 7.6 ± 4.2; 0.8 ± 0.4; 6.8 ± 3.7; and 55.9 ± 25.3) as well as in the 13 patients on dietotherapy with steatorrhea (3.7 ± 0.9; 6.4 ± 2.4; 0.5 ± 0.2; 9.7 ± 4.7; and 38.1 ± 20.2). Values in the 3 treated patients without steatorrhea were normal except for XA (17.6 ± 4.8). Tryptophan loading significantly increased IAA, Kyn, XA and indican in the untreated group (33.2 ± 14.2; 78.8 ± 48.1; 38.9 ± 30.8; and 73.0 ± 24.3), and in the treated patients with steatorrhea (47.2 ± 21.7; 82.1 ± 36.4; 25.2 ± 14.6; and 40.9 ± 27.0). In the 3 treated patients without steatorrhea, Kyn and XA also rose significantly (134.8 ± 23.1; and 52.2 ± 42.4), but IAA and indican did not increase abnormally. Administration of pyridoxine corrected the abnormal excretion of Kyn in all patients and reduced XA, but not to normal. These results demonstrate a deficiency of vitamin B_6 in non-tropical sprue which is clearly accentuated by loading with L-tryptophan.

The Relative Abundance of Viral Receptors: An Explanation of the Differential Susceptibility of Suckling and Adult Mice to Coxsackie B_1 Infection. CALVIN M. KUNIN AND NORMA E. HALMAGYI, Charlottesville, Va. (introduced by William S. Jordan, Jr.)

The marked difference between infants and adults in susceptibility to various virus infections has been the subject of considerable speculation. One explanation for this difference may be found in quantitative variations in the tissue distribution of those specific virus receptor sites which govern the first step of cellular infection. Adsorption technics and plaque count assays were employed in a study of the distribution of Coxsackie B_1 (Conn. 5 strain) receptor site in various rodent tissues. A heat-, ether-, and chloroform-labile sedimentable virus-adsorbing substance was found in noncultured minces and homogenates of brain of suckling and adult mice and rats; it was not found in any guinea pig or rabbit tissues. Suckling mouse brain was much more active in adsorption than adult brain as indicated by dilution and kinetic studies; cortisone treatment did not enhance receptor activity of adult tissues. Multiplication of virus was demonstrated in adult mouse brain in the absence of overt disease. Slight adsorptive activity was demonstrated in mouse squamous cell carcinoma and

teratoma minces, but not in 8 other mouse tumors or in mouse muscle, liver, gut, blood, fetal or maternal tissues. The marked neurotropism of this Coxsackie B_1 strain in suckling mice was thus correlated with the relative abundance of receptor site in CNS tissue. The enhanced susceptibility of cortisonized adult mice to Coxsackie B_1 infections reported in the literature appears to be due to mechanisms other than increased receptor site activity.

Uric Acid Excretion in Gout: Evidence of an Abnormality in Renal Transport. WILLOUGHBY LATHEM* AND GERALD P. RODNAN, Pittsburgh, Pa.

The mechanism of hyperuricemia in gout remains in dispute. Recent evidence has suggested that uric acid transport by the kidney may be altered in gout and that this may contribute to the hyperuricemia. In the present study the renal transport and excretion of uric acid by gouty patients were examined under conditions of acute intravenous urate loading and compared with a group of nongouty individuals studied under similar conditions. Sodium or lithium urate was infused intravenously (16 to 32 mg per minute) over a 2 to 3 hour period while measurements of plasma and urine urate levels were made and the glomerular filtration rate (inulin clearance) determined. Only subjects with normal filtration rates were included. As the plasma urate level increased during the infusion the filtered load and urinary excretion also increased. A distinct impairment in urate excretion was apparent in all of the gouty subjects studied. At comparable plasma levels (8 to 30 mg%) and filtered loads, the gouty subjects excreted a consistently and significantly smaller proportion of the filtered urate load (from ½ to ⅕ less) than the nongouty controls. This was also true of one of two subjects with essential hyperuricemia studied. In ⅔ of the control subjects the difference between the filtered load and the excretory rate (minimum reabsorptive rate) became constant (average, 10 mg per minute) at high plasma levels. In all of the gouty subjects the minimum reabsorptive rate increased progressively and did not become constant, attaining values of 20 or more mg per minute. These results establish that an abnormality in renal urate transport—either increased reabsorption or decreased secretion—exists in gout. This abnormality may be a basic feature of the metabolic disorder of gout or may be acquired or appear as an adaptative response during the course of the disease.

"Complete" and "Incomplete" Pathologic "Cold" Antibody Activities: One or Multiple Antibody Species? JOHN P. LEDDY, NORMA TRABOLD, SCOTT N. SWISHER* AND JOHN H. VAUGHAN,* Rochester, N. Y.

Human sera containing pathologic "cold" antibodies are recognized by two different serological activities: direct agglutination of most human red cells, and, in the presence of complement, sensitization of erythrocytes to reagglutination when the cells are subsequently tested with "non-gamma" antiglobulin sera. The sensitization of erythrocytes for the antiglobulin test has been called "incomplete" cold agglutinin and has been thought possibly to depend upon an antibody different from the direct agglutinin, as in the case of complete (19S) and incomplete (7S) Rh antibody. Four pathologic cold agglutinating sera were studied by a variety of technics designed to separate, if possible, these two activities; separation was not achieved. Both activities were found to reside in protein fractions having the characteristics of 19S γ-globulins. They sedimented together in sucrose density ultracentrifugation, migrated together in starch block electrophoresis, and eluted together from DEAE columns. Both activities were eluted at 37° C from erythrocytes sensitized at 4° C in the absence of complement; incomplete activity was demonstrable only when complement was subsequently added to the eluate. Both activities were susceptible to destruction by mercaptans. Neither activity could be differentially absorbed from the sera. Neither activity was demonstrable when I-negative erythrocytes were employed. The data, therefore, are consistent with the concept that the two agglutinin activities are attributable to the same antibody molecule; the difference is ascribable to the presence or absence of complement when the antibody combines with the erythrocytes. It has been suggested that the interaction of pathological cold agglutinins with complement is important in the pathogenesis of certain acquired hemolytic disorders. Since this "incomplete" antibody activity is not dependent upon a separate, 7S, mercaptan-resistant globulin, these observations provide impetus to the theoretical possibility that treatment of such patients with agents such as penicillamine might be beneficial.

The Simultaneous Measurement of Pressure and Flow in the Pulmonary Arterial System of Man. G. DE J. LEE, R. BOSMAN, A. J. HONOUR, R. M. MARSHALL AND F. D. STOTT, Oxford, England (introduced by A. B. duBois).

A pneumatic flowmeter has been devised for use with the body plethysmograph. It consists essentially of a servo system maintaining a constant pressure within the plethysmograph, and has a linear response from 0 to 200 ml per second, and a uniform frequency response up to 12 cycles per second. Gas mixtures of differing solubility and concentration were inspired by subjects in the plethysmograph to determine the relative importance of mechanical events within the thorax, and pulmonary blood flow itself, on the pattern of pulsatile gas movement within the lungs during each heart cycle. Small, biphasic fluctuations in gas volume occurred with each heart beat, irrespective of the gas breathed. These were compression effects by the heart on neighboring viscera and were trivial compared with the fluctuations superimposed by the effects of pulmonary blood flow, shown when soluble gases were breathed. The uptake rate of a soluble gas, such as nitrous oxide, was proportional to its alveolar concentration and to the pulmonary

capillary blood flow. The pulmonary capillary blood flow was pulsatile with each heart beat, and the right ventricular stroke volume could be calculated from the N_2O uptake curves and expired N_2O concentration measured simultaneously. Comparison between this and the Fick cardiac output estimation was within ±8 per cent. The pulmonary capillary flow pattern conformed closely to the pulmonary artery pressure obtained simultaneously during cardiac catheterization. In the normal subject, peak pulmonary blood flow followed approximately 0.2 second after the peak pulmonary arterial pressure. The effect of pulsatile pulmonary capillary blood flow on oxygen uptake was also studied by continuously recording gas uptake when subjects breathed mixed venous concentrations of carbon dioxide in oxygen. The oxygen uptake was pulsatile and was similar to the N_2O uptake pattern.

Continuous Inulin and PAH Clearance Studies During Prolonged Administration of Deoxycorticosterone (DOC) in Normal Man. JAMES B. LEE AND DAVID P. LAULER, Boston, Mass. (introduced by George W. Thorn).

Prolonged administration of mineralocorticoid to normal man results in retention of sodium, chloride and water followed by an "escape mechanism" whereby urinary sodium progressively rises to equal or even exceed intake. Previous studies have suggested an increase in glomerular filtration rate (GFR) associated with this escape; however, the definitive study of continuously monitored renal hemodynamics through a control period followed by the phase of DOC-induced sodium retention and ultimately through the phase of escape has been lacking. In this study, simultaneous evaluation of renal and metabolic events which occur during adaptation to DOC was made upon a normal subject receiving a constant weighed diet (267 mEq sodium). By means of a constant intravenous infusion the GFR (C_{In}) and renal plasma flow (RPF) (C_{PAH}) were continuously monitored for 3 control days and then, during DOC administration, for 7 consecutive days using six 4-hour collection periods each day. Renal clearances of total solute (C_{Osm}) and solute-free water (C_{H_2O}) were determined every 4 hours together with measurements of urinary electrolytes, acidification parameters, creatinine, urea and catecholamines. The experimental design provided for normal activity of the subject, enabling ambulation within a 40-foot radius. During the control period a marked diurnal variation of both GFR and RPF occurred. Within 24 hours after DOC administration, there was a rapid elevation in mean 24-hour renal plasma flow (744 to 911 ml per minute) followed by a more gradual rise in mean 24-hour GFR (123 to 152 ml per minute). A surprising reduction in RPF 24 hours prior to the "escape" was associated with an increase in C_{H_2O} and an elevation in serum sodium. On the day of "escape," maximal natriuresis was associated with a pronounced rise in renal blood flow with no change in mean 24-hour GFR.

A Metabolic Substitute for Vitamin B_{12}. CARROLL M. LEEVY, HERMAN BAKER, OSCAR FRANK AND HERMAN ZIFFER, Jersey City, N. J. (introduced by Harold Jeghers).

Studies of cyanocobalamin (B_{12}) demonstrate that administered B_{12} partly displaces hepatic stores of this vitamin. The present study was undertaken to determine whether it also displaces other substances from the liver. Under conditions of right hepatic vein catheterization, 100 μg of crystalline B_{12} was given to 8 healthy subjects and 7 patients with inactive cirrhosis. It caused release of B_{12} and a previously undescribed "Factor X" in both groups of patients; no alteration in splanchnic blood flow or oxygen consumption was noted. Unlike B_{12}, Factor X is stable to alkaline hydrolysis at pH 11 to 12, 121° C, 16 pounds per square inch for 30 minutes. It is not naturally present in normal serum or serum from patients with elevated B_{12} due to hepatic necrosis. Thirty minutes after intravenous B_{12}, 1 ml of serum from hepatic venous blood yields an average of 14 mg of lyophilized protein-free alkali-thermostable material; 2 hours after its administration, B_{12} activity remains elevated while Factor X activity disappears. Factor X possesses four times the activity of B_{12} for *Lactobacillus leichmannii*, *Escherichia coli* 113-3, *Euglena gracilis*, and *Ochromonas malhamensis*, the latter organism being the most critical indicator of B_{12} hematopoietic activity. This substance produced two ultraviolet-absorbing spots on ascending chromatography with butanol : acetic acid : water (12 : 3 : 5) which did not correspond to any known purine or pyrimidine moieties. It is concluded that intravenous B_{12} in man triggers hepatic release of an alkali-thermostable factor which permits growth of B_{12}-requiring microorganisms. This factor appears to bypass and completely fulfill the metabolic requirement and function of B_{12}.

Abolition of Digitalis-induced Ventricular Tachycardia by Ryanodine. EDWARD LEONARD AND STEPHEN HAJDU, Bethesda, Md. (introduced by Donald S. Fredrickson).

Ryanodine is a complex water-soluble alkaloid which first evoked interest as the substance responsible for the insecticidal properties of extracts from the plant *Ryania speciosa*. Ryanodine is extremely toxic for mammals. It induces skeletal muscle contracture, and in much smaller concentrations depresses myocardial contractility. In this laboratory it has been observed that ryanodine abolishes the action of cardiac glycosides on contractility of cardiac muscle. In isolated frog heart and rat ventricle strips the effect of cardiac glycosides on contractility can be eliminated or prevented by ryanodine. Because of this digitalis-ryanodine antagonism, studies were undertaken to determine whether ryanodine could reverse digitalis toxicity in intact mammals. In anesthetized cats, otherwise fatal ventricular tachycardia induced by intravenous digitoxin was abolished by ryanodine in all instances within 5 to 15 minutes. The

resultant heart rate after ryanodine tended to be in the normal range, and the cardiac cycle was initiated by the sinus or by another focus which was always supraventricular. The intravenous injection of ryanodine in the amount required to abolish ventricular tachycardia had no effect on the ECG of normal cats. Thus far, similar results have been obtained in the dog. Despite the suppression of ventricular arrhythmias, preliminary experiments suggest that ryanodine may not reverse digitalis-induced conduction defects. Thus the AV block caused by digitalis in a dog was not reversed by ryanodine; and administration of digitoxin to a ryanodine-treated cat caused appearance of AV block without ventricular arrhythmia. Ryanodine, with its capacity to reverse the action of digitalis on ventricular muscle, merits investigation as a specific agent for the reversal of digitalis-induced ventricular arrhythmias.

Direct Measurement of Human Cardiac Excitability. DAVID H. LEWIS, HOWARD F. WARNER AND MARY B. ALLAN, Philadelphia, Pa. (introduced by Truman G. Schnabel, Jr.).

During routine cardiac catheterization opportunity has been taken to quantitate the excitability of the human heart. A wire electrode, insulated except at the tip, was passed through the catheter and advanced until the tip lay within the myocardium. The transition from an intracavitary position to an intramyocardial one was recognized by the change in the recording from the electrode from an intracavitary potential to a form resembling an action potential. Square wave stimuli were delivered to the heart, of varying amplitude (0.1 to 25.0 v) and duration (1 to 15 msec) either as single shocks with a known delay from the R wave or as continuous stimuli applied at varying times in every fourth cycle. In 25 patients, records have been obtained 13 times from the right atrium, 3 from the left atrium, 8 from the right ventricle, and once from the left ventricle. Results from the atrium and ventricle were similar. Threshold was of the order of 0.5 to 4.0 v and rose as the duration was decreased below 7 msec. Absolute refractoriness was noted in the range of 250 to 300 msec after the onset of electrical activity at the point of stimulation. Relative refractoriness was seen but the curve of amplitude vs interval became perpendicular at 1 to 2 v above threshold. Responses were obtained during the recovery phase of the action potential. Prolonged latency in either the absolute or relative refractory period was not seen. Supernormality, with threshold stimuli, was observed, but was the exception rather than the rule. Multiple responses following a single stimulus (vulnerability) were seen in cases with spontaneous premature contractions, but were rare. It was quite pronounced in one instance following a large dose of intravenous digitalis. In both the atrium and ventricle repetitive stimulation reproduced the phenomenon of parasystole.

Porphyrin Synthesis in Thalassemia and Sickle Cell Anemia. HERBERT C. LICHTMAN AND FELIX FELDMAN, New York, N. Y. (introduced by Ludwig W. Eichna).

Normal porphyrin metabolism is a prerequisite for optimal heme synthesis. Hemoglobin production is known to be reduced in sickle cell anemia and thalassemia. The present study was designed to determine whether a particular portion of the pathway for heme synthesis is intact in these hereditary anemias. Measurements were made of the amount of uroporphyrin, coproporphyrin and protoporphyrin synthesized, *in vitro*, from Δ-amino levulinic acid using the circulating erythrocytes of patients with thalassemia and sickle cell anemia as a source of the necessary enzymes. Comparisons were made with normal erythrocytes. Uroporphyrin production was entirely comparable in the three groups. Coproporphyrin production was increased twofold in the thalassemic group compared with the normal or sickle cell anemia groups. Protoporphyrin synthesis was elevated 40- to 45-fold in both anemia groups. An increased number of identifiable immature cells (reticulocytes and nucleated red blood cells) was present in the incubation mixtures derived from the anemia groups. The increase in total porphyrins and especially of protoporphyrins synthesized in the system in the anemia groups may very well be due to the increased numbers of immature cells present. Conclusions: 1) No deficit in porphyrin synthesis, *in vitro*, from Δ-amino levulinic acid could be demonstrated using thalassemia or sickle cell anemia erythrocytes as enzyme source. 2) A marked increase in total porphyrin synthesis was found, especially of protoporphyrin, in the incubation mixtures containing erythrocytes of thalassemia and sickle cell anemia patients.

Removal of Damaged Cells from Intestinal Mucosa. MARTIN LIPKIN, H. OLIVER BROWN AND MILTON L. LEVINE, New York, N. Y. (introduced by Thomas P. Almy).

Nuclei of rapidly proliferating jejunal epithelial cells of mice were labeled by injection of C^{14}-thymidine. Animals were sacrificed at varying intervals thereafter, and specific activity determined on mucosal precipitates containing DNA. In parallel experiments utilizing H^3-thymidine, tissues were prepared for study by microradioautography. In control animals, the specific activity of the incorporated thymidine began to decline 35 to 40 hours after injection. Microradioautographs demonstrated that this interval was required for the normal column of labeled epithelial cells to migrate from the crypts to villus tips and begin extrusion. In the experimental group, animals were injected simultaneously with colchicine and labeled thymidine. Thus, crypt cells were labeled and then damaged as they underwent mitosis. The decline in specific activity in this group began in 6 hours; 30 hours after injection, one-quarter of the label had been removed from the mucosa

of the experimental group, while there was no loss of label from the controls. Microradioautographs revealed that labeled cells with colchicine-induced damage were removed from the crypt column beginning within 6 hours after injection. Progressive loss of these cells was noted thereafter, and the cells appeared to be extruded directly from the crypt lumen, preventing migration onto the villi. Following removal of the damaged cells, the villi and remaining crypt and villus epithelial cells appeared normal. The study demonstrates a method of response of intestinal mucosa to damage induced in a limited number of regenerating cells. The possible role of this response in the production of ulceration of the gastrointestinal tract will be further evaluated.

Eosinophilia as a Consequence of Antigen-antibody Interaction. MORTIMER LITT, Boston, Mass. (introduced by B. D. Davis).

Eosinophilia occurs in such a wide variety of pathological situations that it has seemed as if no common mechanism underlies the phenomenon. The peritoneal cavity of guinea pigs was used as a site for testing the eosinophilotactic potency of various materials, and the results indicate that eosinophilia is related to a particular phase of the immune response. Exudates were obtained by lavage through an abdominal trocar and the total population of cells characterized by standard hematologic techniques. After active sensitization, by repeated weekly injections of foreign protein, eosinophilia appeared within 24 hours after injection of the antigen. Passive transfer of tissue (peritoneal lining or lung), tissue extracts, or serum from sensitized animals produced eosinophilia, provided that the sensitizing antigen accompanied the material transferred, and that the recipients had been actively sensitized with a noncross-reacting protein. Washed immune precipitates were equally effective; these consisted of either bovine serum albumin with rabbit antibody, or hemocyanin with guinea pig or rabbit antibody. Such mixtures were effective in antigen excess, equivalence and antibody excess. The activity of the antisera disappeared after absorption with specific antigen. Thus, the active transfer factor in serum seems to be antibody and the active material *in vivo* appears to be an immune complex. The present findings appear to account for the common association of eosinophilia with hypersensitive states, since immune complexes also initiate various hypersensitive reactions. In addition, it appears likely that during the recuperative phase of an infectious disease, the eosinophilia, which is so frequently seen, signals the beginning production of antibody.

A New Method Suitable for Clinical Measurement of Cerebral Circulation Time. W. D. LOVE, L. P. O'MEALLIE AND W. W. LEMMON, New Orleans, La. (introduced by G. E. Burch).

When a bolus of radioisotope circulates through the brain, the time course of cerebral radioactivity resembles that in arterial blood. However, because of the time required for isotope to traverse vessels within the area monitored, the cerebral curve has a lower, later peak and a more gradual downslope. The differences between these curves have been used as the basis for calculating mean cerebral circulation time (MCCT). The clinical advantages of this "wave distortion" method of analysis are 1) MCCT can be measured without carotid or jugular puncture, and 2) differences in cerebral circulation to separate areas can be determined. In a model, radioactivity was recorded by virtually lag-free counters which monitored inflowing and outflowing fluid and the "brain" during passage of a radioactive bolus. True MCCT was equal to the difference between circulation times to the inflow and outflow points. MCCT was estimated from inflow and organ curves by two methods. One, an empirical procedure, was based on the difference in location of the median point in the two curves. The second was a curve-fitting technique. In a range of circulation times from 4.1 to 23.4 seconds there was a mean ± 0.2 second, or ± 1.6 per cent difference between MCCT calculated by these methods and the actual value. Similar studies were done in 13 anesthetized dogs with the cranium exposed surgically for monitoring. Radioactivity in the infraorbital artery and torcular Herophili was continuously recorded by aspirating blood through tubing under monitors. One carotid and both vertebral arteries were ligated. I^{131}-albumin was injected proximal to a mixing chamber in the other carotid. MCCT values in 53 determinations were manipulated to range from 0.9 to 30.0 seconds. Mean difference between the inflow-outflow and inflow-organ techniques was ± 1.4 seconds, or ± 16.6 per cent.

Relationship of Sulfhydryl Inhibitors and Reductive Conditions to the Action of Insulin on Adipose Tissue. W. S. LYNN, JR.,* EUGENIA EARNHARDT AND ROSE BROWN, Durham, N. C.

Recent studies by Langdon have implied that reduced insulin may be the active form of the hormone. Schwartz and co-workers also have hypothesized that antidiuretic hormone probably exerts its effects through a disulfide exchange reaction. We, therefore, have studied the effects of -SH inhibitors as well as reducing conditions on rat adipose tissue, in the presence and absence of insulin. Both iodoacetate and parachloromercuribenzoate (PCMB) (2×10^{-5} M) mimic the effects of insulin on this tissue *in vitro;* i.e., more glucose 6-C^{14} (5-fold) is converted to fatty acids and more glucose 1-C^{14} to CO_2. O_2 uptake and lactic acid production are slightly reduced, tissue content of ATP and G6P increases, and the tissue content of glucose and water decreases in the presence of these -SH inhibitors, as well as insulin. The effect of insulin, along with -SH inhibitors, is additive. Furthermore, under strong reductive conditions (i.e., N_2 and HCN or N_2 and H_2S) insulin, PCMB, or iodoacetate potentiates the effects of these reductive conditions on lactate production and fatty acid synthesis from glucose or butyric acid. Thus, sulfhydryl inhibitors produce the

same effects as insulin. Insulin potentiates the effects of -SH inhibition, and insulin and -SH inhibitors potentiate the effects of strong reducing agents. None of these agents or conditions is lipolytic. The primary action of insulin thus appears to be related to the easily altered sulfhydryl content of this tissue, and may act, like PCMB, by reacting with tissue -SH groups.

Protein-Bound Sulfur (PBS); Its Role in Inhibiting Thyroid Protein-bound Iodine. F. MALOOF AND M. SOODAK, Waltham and Boston, Mass. (introduced by J. Lerman).

A cytoplasmic particulate system which transfers the sulfur of thiourea to protein (PBS) in the presence of thiocyanate ion (10^{-3}M) and DPNH (10^{-3}M) has been isolated from animal or human thyroid tissue. The inhibition of the *in vitro* formation of protein-bound iodine (PBI) by thyroid particulate protein requires a 20-fold greater concentration of cysteine (10^{-4}M) than thiourea (5×10^{-6}M), although the reduction potential (Eo') of cysteine, -0.15, equals that of thiourea, Eo' $= -0.21$. Incubation of S^{35}-labeled cysteine (10^{-4}M) with the thyroid particulate protein produces only one-tenth as much PBS as thiourea even with thiocyanate and DPNH. This suggests that PBS formation is important in inhibiting PBI. The stimulation of *in vitro* PBI formation by various peroxide-generating systems suggests that a peroxidase is involved in iodination. This has led to the assumption that thiourea acts by inhibiting peroxidase. Thiourea (5×10^{-5}M) or iodide was incubated with thyroid protein plus flavin mononucleotide (FMN), in the light, as a peroxide-generating system. FMN (10^{-5}M) leads to the formation of PBS (10 mμmoles) equal to that of the thiocyanate-DPNH system. However, the FMN and the thiocyanate-DPNH system differ, since the former is inhibited by dark, by thiocyanate (10^{-3}M), and is only partly (60 per cent) inhibited by heat ($100° \times 3$ minutes). PBI formation in the presence of FMN is about 1 mμmole. Preincubation of the particulate protein with sulfite (0.01 M), a compound known to cleave disulfide bonds specifically, inhibits the subsequent formation of PBS or PBI by FMN. These data lead to the following conclusions. A disulfide bond in thyroid tissue is essential for the formation of both PBI and PBS by FMN. Thiourea cleaves this disulfide bond to form PBS. The action of thiourea in inhibiting PBI formation in the presence of a peroxide-generating system may be due to the formation of PBS.

Active Transport of Iron In Vitro by Duodenal Segments and the "Mucosal Block." JAMES G. MANIS AND DAVID SCHACHTER,* New York, N. Y.

Everted segments of rat duodenum transport iron *in vitro* against concentration gradients from fluid bathing the mucosa to fluid bathing the serosa. The active transfer is observed only in the proximal small intestine, is dependent on oxidative metabolism and is limited in capacity. Two steps are involved in the net transport across the gut. 1) The initial and more rapid step is uptake at the mucosal surface of either Fe^{++} or Fe^{+++}; 2) the subsequent transfer to the serosal surface is less rapid, rate-limiting, and relatively specific for Fe^{++}. The active transport mechanism in the duodenum is markedly influenced by pregnancy, by inflammation, and by prior doses of iron. Duodenal segments from rats in the third week of pregnancy transfer iron much more readily than do segments from nonpregnant female controls. Intramuscular injection of turpentine into a hind limb is followed in 2 to 4 days by depression of the iron transport. Administration by gastric tube of 0.4 to 4.0 mg of iron (as $FeSO_4$ or $FeCl_3$) decreases markedly the net transport of iron across the gut *in vitro*. The inhibition can be observed 1.5 hours after the dose and persists for approximately 24 hours. Prior doses of iron salts decrease both steps of the active transport mechanism *in vitro*. The inhibition of mucosal uptake corresponds to the "mucosal block" suggested by experiments *in vivo*, whereas the inhibition of serosal efflux is a new observation which suggests the more appropriate term "transport block." The "transport block" is apparently a direct effect of iron salts on the duodenum. Prior incubation of duodenal segments in media containing either Fe^{++} or Fe^{+++} decreases the net transport of iron observed subsequently.

Evidence for Heterogeneity Among Subjects with Glucose-6-phosphate Dehydrogenase Deficiency. PAUL A. MARKS,* RUTH T. GROSS AND JULIA BANKS, New York, N. Y.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been detected, in varying incidence, in many population groups around the world. The trait appears to be due to a sex-linked gene of intermediate dominance. Previous studies in this laboratory indicated that mutant subjects vary in the severity of the enzyme deficiency and in the thermostability of the G6PD. A comparison has now been made of the electrophoretic mobility and the properties of the catalytic site of G6PD purified 2,000-fold from red cells of 6 normal and of 12 mutant subjects. The mutant subjects varied in expression of this trait as measured by the red cell G6PD activity (RBC-G6PD). The following results were obtained. 1) G6PD of 1 female and 2 males of an Italian family differed from normal G6PD in having a faster electrophoretic mobility and a decreased affinity for TPN. These male mutants were distinguished from other mutant Caucasian males studied in that RBC-G6PD was decreased only 50 per cent. 2) G6PD of a male of Negro-Caucasian parentage (RBC-G6PD decreased 90 per cent) had a lower affinity for TPN, but was similar to normal G6PD in the other properties studied. 3) G6PD of 4 Negro males (RBC-G6PD decreased 85 per cent), 1 Negro female (RBC-G6PD decreased 50 per cent), 1 Caucasion male and 1 female (RBC-G6PD decreased 95 to 98 per cent) all without anemia, and 1 Caucasian male with congenital nonspherocytic hemolytic anemia (RBC-G6PD decreased 98 per cent) were similar to normal G6PD in the prop-

erties of the catalytic site and in electrophoretic mobility. These data indicate that an altered enzyme is present in certain mutant subjects. In addition, G6PD deficiency in man appears to be genetically, as well as phenotypically, heterogeneous.

Effect of Proteolytic Enzymes on Red Cell Rh_0 (D) Content. S. P. MASOUREDIS,* San Francisco, Calif.

The effect of proteolytic enzymes on the Rh_0 (D) red cell content was studied with incomplete I^{131} anti-Rh_0 (D). Aliquots of an R^2R^2 RBC suspension were treated at 37° C with 0.3 per cent trypsin for 10 minutes and with 0.01 per cent papain for 30 minutes. Controls consisted of the same untreated R^2R^2 cell and an rr cell subjected to the same enzymatic treatment. Papain-treated RBC took up 23 per cent more I^{131} than the untreated cells and the trypsin-treated RBC bound 15 per cent more I^{131}. This difference between the enzyme-treated Rh_0 (D) positive cells and the untreated cells was significant at the 5 per cent level, whereas the difference between the two enzymes was not significant at this level. The Rh_0 (D) negative cell took up less than 10 per cent of the I^{131} bound to the R^2R^2 cell and there was no difference between the I^{131} bound to the enzyme-treated and untreated RBC. To test the specificity of the additional I^{131} bound to the enzyme-treated Rh_0 (D) positive cell, the cells were hemolyzed with distilled water and the stroma bound I^{131} was determined after washing the stroma with distilled water. The stroma of the untreated Rh_0 (D) positive cell contained 90 per cent of the total cell bound I^{131} and the stroma of the enzyme-treated cells contained 97 per cent of the original I^{131} bound to the intact cell. Only 45 per cent of the I^{131} was recovered in the stroma of the Rh_0 (D) negative cell after hemolysis. These results indicate that enzymatic treatment of Rh_0 (D) positive red cells with papain or trypsin exposes additional Rh_0 (D) antigen sites on the red cell stroma that are not available in the untreated cell. It appears that the agglutinability of these enzymatically treated cells is due to an increase in antigen sites which in some fashion yields an adequate structure for agglutination.

Dominant Role of Pulmonary Vascular Engorgement as a Determinant of Dyspnea. H. PAGE MAUCK, JR. AND WILLIAM SHAPIRO, Richmond, Va. (introduced by John L. Patterson).

The present observations explored the pathogenesis of dyspnea in patients with and without the rapid induction of pulmonary engorgement. Pulmonary wedge pressure and arterial gas tensions were measured in 13 subjects, 11 with primary systemic hypertension and left ventricular enlargement, one with mitral stenosis, and one with obstructive pulmonary emphysema. Dyspnea was induced in the supine position by breath-holding or exercise on a recording ergometer. Precise correlations were obtained by patient signals at the onset of discomfort (later described). Four patients exercised without developing dyspnea, wedge pressure rise or gas tension abnormality. Dyspnea during breath-holding was experienced by 7 patients without wedge pressure rise but after a 13 mm Hg fall in P_{O_2} and a 6 mm Hg rise in P_{CO_2}. In contrast, 5 hypersensitive patients who exercised maximally (532 kg-m per minute for 2 minutes) experienced dyspnea after a mean rise in wedge pressure of 20 mm Hg, even though P_{O_2} had risen 10 mm Hg and P_{CO_2} fallen 2 mm Hg. The actual wedge pressures at the onset of dyspnea were in the narrow range of 27 to 34 mm Hg, and directional changes in gas tensions were consistent. The patient with emphysema exercised for 4.5 minutes at 493 kg-m per minute and became dyspneic when the wedge pressure had risen from 5 to 15 mm Hg, the arterial P_{O_2} had fallen from 60 to 46 mm Hg, and the P_{CO_2} had risen from 70 to 79 mm Hg. Arterial pH changes were small in all experiments. The similarity of the descriptions of dyspnea by the different patients was notable. These observations are consistent with the hypothesis that the onset of dyspnea is related to the summated effects of multiple respiratory stimuli. When present, pulmonary vascular engorgement plays a dominant role, with 30 mm Hg as the apparent critical level of a rising wedge pressure during exercise.

Tolerance to Bacterial Endotoxin Induced by Experimental Pyelonephritis. WILLIAM R. MCCABE, Chicago, Ill. (introduced by Harry F. Dowling).

Tolerance to the toxic effects of the endotoxins of gram-negative bacteria is a thoroughly documented phenomenon that is readily produced by repeated injections of endotoxin. It has not been observed to occur during clinical or experimental infections. Comparison of the clinical courses of 173 patients with gram-negative septicemia suggested that the relatively mild course exhibited by patients who had concomitant chronic pyelonephritis might be a manifestation of tolerance to endotoxin and prompted the present investigation of the pyrogenic effect of bacterial endotoxin in pyelonephritic rabbits. Pyelonephritis was produced by temporary ureteral ligation followed by the intravenous injection of *E. coli* (0:111,B:4) while weight-matched controls received a similar injection of heat-killed bacteria. Two weeks after the induction of infection, both groups of animals were challenged with 0.5, 1.0, and 2.0 μg of *S. enteritidis* endotoxin (Difco) and fever curves were determined. Rabbits with active pyelonephritis not only exhibited a significant diminution ($t_{24} = 7.91$; $p < 0.001$) in the total magnitude of fever (mean 12.6 ± 6.6) as compared with controls (mean 43.7 ± 10.2) but also demonstrated the classical tolerant fever curve in which a primary temperature rise without a subsequent secondary elevation was observed. Prior administration of thorium dioxide (Thorotrast) abolished the tolerant state and induced a febrile response analogous to that observed in the control animals. These results indicate that proliferation of gram-negative bacteria in the kidney is capable of inducing tolerance to heterologous endotoxin.

Immunization of Man with Globulin-modified Measles Vaccine. FRED R. MCCRUMB, JR., SCHELDON KRESS, RICHARD B. HORNICK, MERRILL J. SNYDER AND ANN E. SCHLUEDERBERG, Baltimore, Md. (introduced by Charles L. Wisseman, Jr.).

Attenuated measles-virus vaccines propagated in canine renal or chick embryo cell culture were used to imunize several hundred susceptible children. Study of routes of immunization revealed that amounts of virus minimally infective for tissue culture were highly immunogenic in man upon parenteral administration. Aerosolized virus also resulted in a high take rate as evidenced by appearance of antibody following vaccination. Intranasal vaccination was associated with widely variable take rates without apparent explanation. Clinically overt reactions to parenterally inoculated vaccines were not invariably benign; 20 to 30 per cent of vaccinees developed fever in excess of 103° F. Respiratory manifestations and constitutional symptoms were uncommon and cutaneous eruptions were those of a modified infection. Less reaction was observed following respiratory administration of vaccine. Despite this reduction in reaction rate, the authors concluded that attenuated measles-virus vaccines were not sufficiently innocuous for large-scale use. Virus did not spread spontaneously to those susceptible children in intimate contact with vaccinees. However, viremia and pharyngeal shedding of the vaccine strain were demonstrated in a few instances. Specific neutralizing and complement-fixing antibody appeared in high titer after administration of attenuated viruses. The slow degradation of these antibodies suggested that immunity would be of long duration. Immunized children were resistant to naturally occurring measles 10 and 15 months after administration of vaccine. The reaction to administered vaccines was reduced by modifying the induced infection with human γ-globulin. Marked decrease in the incidence of significant reaction without alteration of immune response suggests that this method is applicable to large-scale immunization. The paradox of virus and antibody interacting to the advantage of the host without impairment of antigenicity is of some interest. Factors presumably operative under these conditions are discussed.

Differentiation of Nervous and Hormonal Control of Renal and Limb Vascular Beds Utilizing Bretylium and Guanethidine. JOHN C. MCGIFF, Philadelphia, Pa. (introduced by Calvin F. Kay).

Simultaneous and continuous measurements of renal and limb (femoral) blood flows (venous outflow rotameter) of the anesthetized (chloralose) dog during increased intracranial pressure permit observation of enhanced sympathetic vasoconstrictor activity and the alteration of this activity by agents blocking sympathetic nerves (bretylium and guanethidine). In 9 dogs increased intracranial pressure unmodified by these drugs is followed by a striking pressor response (105 per cent of control), a reduction of limb (mean, 40 per cent) and renal (mean, 53 per cent) blood flows, the latter occasionally falling to zero. Renal vascular resistance is augmented (mean, 360 per cent) more than femoral vascular resistance (mean, 240 per cent). The pressor response is reduced within 20 to 40 seconds by bretylium (2 to 7 mg per kg). Concomitantly, renal and femoral flows are always increased. Correspondingly both femoral and renal resistances are reduced, the former much more than the latter. This difference in regional resistance changes is presumably due to continued action of catecholamines (unopposed by bretylium) to which the renal bed is demonstrably more sensitive. Guanethidine has effects similar to bretylium in reducing sympathetic vasoconstrictor activity. In the absence of increased intracranial pressure, the resting anesthetized dog responds to intravenous administration of bretylium or guanethidine with hypotension within 1 minute, which may be preceded by a transient hypertension. Femoral flow increases sharply (from 21 to 330 per cent), whereas renal flow is reduced, although less so than blood pressure; thus, renal resistance is reduced. The opposite effect on regional blood flows is attributed to the drug's unmasking a greater degree of sympathetic tone involving the vessels of skeletal muscle than the vessels of the kidney in the "resting" state. Renal resistance, but no femoral resistance, is increased if an initial hypertensive phase is elicited. This latter effect is presumably related to the liberation of levarterenol to which the kidney is more sensitive.

The Effects of Squatting on the Circulation. MALCOLM B. MCILROY* AND THOMAS V. O'DONNELL, San Francisco, Calif.

The effects of assuming the squatting position on the arterial oxygen saturation, pulse rate and arterial blood pressure have been studied in normal subjects and patients with congenital heart disease. The changes in saturation were followed with an ear oximeter and the arterial pressure usually measured directly in the brachial artery. The following explanation is proposed for the symptomatic relief often seen on squatting in patients with Fallot's tetralogy. 1) Squatting raises the peripheral systemic resistance and increases the systemic venous return by removing the distending force of gravity upon the arteries and veins below the heart. As a result, more of the right ventricular output is directed to the lungs, and the volume of blood shunted into the aorta is reduced. 2) The increase in cardiac output and blood pressure seen on squatting is inversely proportional to the volume of blood in the central reservoirs of the heart and lungs. 3) Squatting produces a marked increase in the arterial oxygen saturation only in patients with a small "central blood volume." 4) The anatomy of the heart in Fallot's tetralogy, with a right ventricular-to-aortic shunt, provides a situation in which small changes in the parallel resistances of the pulmonary stenosis and the systemic arterial bed have a great effect on the arterial oxygen saturation.

Studies on the Effect of Inosine on the Metabolism of Blood Cells During Storage at 4° C. THOMAS J. MCMANUS, Boston, Mass. (introduced by John G. Gibson, 2nd).

The effect of two different anticoagulant solutions, with and without the addition of inosine prior to collection, on the carbohydrate and phosphate metabolism of blood cells during 35 to 42 days of storage at 4° C has been studied. The solutions were acid-citrate-dextrose (ACD), citrate-phosphate-dextrose (CPD), and ACD and CPD with inosine added (ACDI and CPDI) in amounts sufficient to give a final concentration of 2 g per 500 ml of blood. In the presence of inosine, glucose utilization in both solutions was significantly inhibited, whereas lactate production did not vary from the controls. Phosphate ester hydrolysis is initially retarded in CPD compared to ACD, not reaching its maximal rate until the second week of storage. In ACDI and CPDI, this ester pool is much more stable and does not begin to break down to any large extent until after the third week of storage. After 5 to 6 weeks of storage, the gross intracellular phosphate partition in all the solutions appeared similar whether inosine was present at the time of collection or not. CPD remained less acid than ACD throughout storage with an essentially parallel fall in pH. However, CPDI became acid more rapidly than ACDI, resulting in the same pH in both bloods by the fourth week. Pentose and hypoxanthine concentrations leveled out after 35 days, suggesting that the contribution of the nucleoside to carbohydrate and phosphate metabolism had ceased, due to its essentially complete utilization. We conclude that the addition of inosine to the anticoagulant solution prior to collection is probably not the optimal procedure for utilizing this metabolic adjuvant. The amount of purine that is shown to accumulate in inosine-treated blood, however, represents a serious hazard to patients with impaired renal function.

Simultaneous Change in Hepatic Glucose Utilization, Hepatic Glucose Production and Hepatic Glucose Balance During Glucose Loading. DAVID MEBANE, FRANK LECOCQ, BURTON COMBES AND LEONARD L. MADISON,* Dallas, Tex.

Since net hepatic glucose output equals hepatic glucose production minus hepatic glucose utilization, changes in one or both of these parameters may account for the decrease in hepatic glucose output that attends glucose loading. In these studies changes in hepatic glucose balance, hepatic glucose production and hepatic glucose utilization were measured simultaneously during glucose loading in 5 dogs with portacaval shunts, a preparation permitting measurement of hepatic rather than splanchnic glucose balance. Hepatic glucose balance was determined every 15 minutes for 160 minutes from measurements of hepatic blood flow (Bradley's technic) and hepatic venous-arterial glucose concentration differences. Hepatic glucose production was determined from changes *across the liver* in the specific activity of blood glucose labeled by a constant infusion of glucose-U-C^{14}. Hepatic venous and arterial glucose specific activities were determined by the gluconate method of Blair and Segal. Hepatic glucose utilization is the difference between hepatic glucose and hepatic glucose balance. During control periods, hepatic glucose production and hepatic glucose output were identical (72.7 and 72.1 mg per minute), thereby indicating the absence of hepatic glucose utilization during fasting. Glucose infusions (3 to 12 mg per kg per minute) resulted in a decrease in hepatic glucose output consequent to both a decrease in hepatic glucose production and an increase in hepatic glucose utilization. The 72 per cent decrease in hepatic glucose output was the result of a 51 per cent decrease in hepatic glucose production from 72.7 to 35.3 mg per minute and a simultaneous increase in hepatic glucose utilization from 0 to 15 mg per minute. These data indicate for the first time that the decrease in hepatic glucose output which follows glucose loading is the consequence of both an increase in hepatic glucose utilization and a decrease in hepatic glucose production.

Alterations in Steroid Hormone Secretion Produced by Inhibition of Cholesterol Biosynthesis. JAMES C. MELBY, MARLYN ST. CYR AND S. L. DALE, Little Rock, Ark. (introduced by Richard V. Ebert).

Interference in the formation of cholesterol, a precursor of the steroid hormones, might be expected to result in reduced steroid hormone production and/or emergence of noncholesterol pathways of steroid formation. To study this effect triparanol, a triphenylethane derivative which inhibits desmosterol conversion to cholesterol, was given laboratory animals and human subjects in dosage sufficient to reduce net cholesterol synthesis. Steroidogenesis *in vitro* was assessed by incubation of adrenal slices obtained from rats pretreated with triparanol and from controls. Acetate-1-C^{14} or mevalonate-2-C^{14} was added to all and corticotropin to half of the incubates. Corticosterone, deoxycorticosterone and aldosterone were separated by paper chromatography. Eluates were divided for quantitation and scintillation counting. Pretreatment with triparanol reduced corticosteroid production by 50 to 75 per cent. Specific activities of individual corticosteroids, however, demonstrated a 2- to 10-fold increase over controls. Alterations of steroid specific activity in corticotropin-stimulated preparations suggest the existence of a noncholesterol pathway of steroid synthesis. Triparanol in a dose of 1,000 mg per day for 10 days significantly reduced cortisol and aldosterone production of healthy human subjects. Impairment of adrenal secretory responsiveness to corticotropin stimulation and to pyrogen stress was demonstrated. Remission of features of primary aldosteronism, Cushing's syndrome and adrenal virilism was associated with a curtailment of corticosteroid and androgen production in 4 patients receiving triparanol. Testicular androgen production was unaffected by triparanol administration in a male with Addison's disease

and in healthy adult males during exogenous corticosteroid-induced adrenal suppression.

Physiological Implications of Changes in Oxyhemoglobin Dissociation. W. F. MILLER, R. L. JOHNSON, JR. AND J. D. O'KEEFE, Dallas, Tex. (introduced by Carleton B. Chapman).

A re-evaluation of previous studies from this laboratory showed large shifts to the right of the O_2-Hb dissociation curve in normal subjects and patients with mitral stenosis during severe exercise, and both at rest and during exercise in severely anemic patients. Only part of these shifts can be explained by pH changes. The magnitude of these shifts at the 50 per cent saturation level are: normal 15 mm (12 mm pH-dependent), anemia 17 mm (10 mm pH-dependent), mitral stenosis 10 mm (7 mm pH-dependent) $p < 0.02$. It is often stated that these shifts aid diffusion of O_2 to the tissues, but only Keys and co-workers have attempted to quantify such effects. If tissue diffusion is a limiting factor in O_2 exchange, the rate of exchange would be a function of the mean capillary O_2 tension, a factor not considered by Keys' group. With our data obtained at maximal O_2 intake and employing a dynamic mathematical analysis, estimates of maximal resistance to diffusion in exercising muscle mass (mm Hg per L per minute) were obtained: normal 14, anemia 25, mitral stenosis 28. With these, a graphic analysis yields estimates of the maximal increase in O_2 intake resulting from observed shifts: normals 9 per cent, anemia 10 per cent, mitral stenosis 3 per cent. According to the method of Keys' group, the increase in our anemic patients at maximal O_2 intake would be 21 per cent. Since, in the present analysis, O_2 tension of mixed venous blood from the exercising extremity was assumed to be the same as end-capillary O_2 tension in the exercising muscle, the actual resistance to diffusion in muscle is probably less than our estimates. Thus, the importance of shifts in the O_2-Hb dissociation, during heavy exercise, in facilitating O_2 uptake is small.

Studies on the Blood-cerebrospinal Fluid Barrier in Hepatic Failure. EDWARD W. MOORE, GEORG W. STROHMEYER AND THOMAS C. CHALMERS,* Boston, Mass.

Animal studies indicate that the distribution of ammonia across membranes is pH-dependent, according to non-ionic diffusion theory. In hepatic failure, however, correlations between ammonia concentrations, pH, and neurological disturbances are often lacking, suggesting abnormality in the blood-CSF barrier. Functional integrity of the barrier was studied by comparing blood and CSF concentrations (after intravenous infusion) of antipyrine, sufadiazine and *p*-aminohippurate (PAH)—compounds distributed according to non-ionic diffusion theory. Plasma drug concentration was maintained constant after priming, and CSF obtained at intervals for 8 hours through an indwelling spinal catheter. CSF/plasma equilibrium ratios for antipyrine and PAH (12 patients) and sulfadiazine (7 patients) were predictable from existing pH gradients and were similar to those previously reported in subjects without liver disease. In sharp contrast, steady state CSF/arterial ammonia ratios were independent of pH gradients, and CSF ammonia was linearly related to arterial ammonia over a wide range of concentrations. Because this linear function did not intercept zero, the CSF/arterial ammonia ratio rose with increasing arterial concentrations, being significantly greater in patients with cirrhosis. The difference between expected CSF/arterial ratios (calculated from existing pH gradients) and observed ratios was inversely related to arterial ammonia concentration. Although steady state CSF/arterial ammonia ratios showed no correlation with pH gradients, alteration of gradients by drug infusion qualitatively changed these ratios in the expected direction. In conclusion, the functional integrity of the blood-CSF barrier for partially ionized compounds is intact in cirrhosis. That CSF ammonia is linearly dependent on arterial concentration irrespective of pH suggests artifact in the methodology (Nathan-Rodkey and Conway) or binding sufficient to obscure the pH gradient effect. The proportion of artifact appears to decrease at higher arterial concentrations, probably accounting for the closer correlations noted in animal studies and also for the fact that only qualitative changes in distribution ratios occur in response to pH change.

Extreme Sensitivity of Parathyroid Hormone Secretion and of Renal Response to Phosphate Infusion. PETER A. F. MORRIN, ERIC REISS AND W. BURTON GEDNEY, St. Louis, Mo. (introduced by Neal S. Bricker).

In studies of renal phosphate excretion it is commonly assumed that alterations in parathyroid hormone secretion induced by changing plasma phosphate concentrations require no special consideration. This assumption has been examined critically in the dog. In euparathyroid dogs, increasing the plasma phosphate only 0.5 to 1.0 mg% by slow phosphate infusion resulted in doubling to tripling of the fraction of filtered phosphate excreted (C_{PO_4}/GFR) while the apparent phosphate reabsorption rate remained unchanged. A constant phosphate reabsorption is expected only at T_m whereas these experiments were performed at plasma phosphate levels (4.6 to 6.0 mg%) of one-half to one-third that required to saturate tubular reabsorptive capacity. When a high C_{PO_4}/GFR has been attained by a slow phosphate infusion, administration of calcium intravenously promptly restored this ratio to or below baseline values. Calcium exerted this effect even though its infusion caused further elevation of plasma phosphate. When the procedure was reversed and calcium infused from the beginning, phosphate infusion resulted in a constant C_{PO_4}/GFR and an increasing rate of apparent phosphate reabsorption. The specific effect of calcium on parathyroid secretion was demonstrated by a parathyroidectomized dog given parathryroid extract intravenously at a constant rate. In this experimental preparation calcium had little effect on C_{PO_4}/GFR. Most studies

were performed in dogs with unilateral renal disease. As in previous studies with this experimental model, the pattern of phosphate excretion in disease and control kidneys was closely comparable under all conditions examined. These experiments demonstrate unusual sensitivity of the mechanisms responsible for parathyroid hormone release. Distinction cannot be made between a direct stimulatory effect of minimal hyperphosphatemia on parathyroid secretion and an effect mediated by slight depression of ionized calcium. The evidence presented also indicates that parathyroid hormone regulates C_{PO_4}/GFR at all levels of filtered phosphate.

Refined Antihypertensive Medullorenal Extract and the Protective Action of the Kidney Against Hypertension. E. E. MUIRHEAD,* J. W. HINMAN, E. G. DANIELS, M. KOSINSKI and B. BROOKS, Detroit and Kalamazoo, Mich.

Renoprival hypertension of the dog occurs consistently when the intake includes sodium and dietary protein. This hypertension is ameliorated by a renal homotransplant. Uretorocaval anastomosis, under identical circumstances, is not conducive to hypertension. Finely divided whole kidney and renal medulla explanted to the peritoneum and lungs, respectively, prevent the hypertension. A nontoxic crude extract of renal medulla prevents this hypertension. The extract decelerates the evolution of renoprival hypertension when massive sodium loads are interposed (Na intake 7.5 to 15 mEq per kg per day), thus yielding results similar to those of ureterocaval anastomosis. Based on the above, it has been suggested that the protective action of the kidney against hypertension resides in the medulla and that this function may be extractable as a principle. The medullorenal extract fails to prevent the renin pressor effect but prevents renoprival hypertension when daily renin pressor peaks are superimposed on nephrectomy plus protein and sodium (slope Δ BP vs zero, p 0.65). Nonrenal tissue extracts under these conditions are not protective against hypertension (slope Δ BP vs zero, $p < 0.001$). The medullorenal extract yields results similar to those of intact kidneys. Utilizing ethanol fractionation with and without prior dialysis, the medullorenal extract was separated into four fractions: 1) The 50 per cent ethanol insoluble fraction was ineffective (BP vs renoprival state, p 0.9; vs medullorenal extract, $p < 0.02$). 2) The 10 per cent ethanol insoluble fraction yielded partial protection against renoprival hypertension (BP vs renoprival state, $p \backsim 0.05$; vs extract, p 0.15). 3) The dialysate was inconclusive, seemingly effective and ineffective lots being derived. 4) The supernatant solution after 50 and 55 per cent ethanol precipitation was consistent in preventing renoprival hypertension (vs renoprival state, $p < 0.001$; vs extract, $p > 0.9$). The latter fraction contains 2 to 3 mg organic solids per ml. The crude extract and fractions are not pyrogenic to rabbits. It would seem that the antihypertensive factor of the renal medulla toward renoprival hypertension has been considerably refined and concentrated.

The Mechanism of Aldosterone Stimulation Following Hemorrhage in the Hypophysectomized Dog. PATRICK J. MULROW AND WILLIAM F. GANONG, New Haven, Conn. and San Francisco, Calif. (introduced by J. W. Hollingsworth).

In order to study how hemorrhage stimulates mainly aldosterone secretion by the adrenal cortex of hypophysectomized dogs, 10 hypophysectomized dogs were nephrectomized and then acutely bled. Control and posthemorrhage samples of adrenal vein blood were collected in consecutive periods. Aldosterone, corticosterone and 17-hydroxycorticoids were measured. In a previous study, in all 10 hypophysectomized dogs, hemorrhage had increased aldosterone secretion (mean secretory rates: control, 10; posthemorrhage 17 mμg per minute; $p < 0.01$). In 8 of 10 hypophysectomized, nephrectomized dogs, hemorrhage failed to stimulate aldosterone secretion (mean secretory rates: control, 3.4; posthemorrhage, 5 mμg per minute). Glucocorticoid secretion was unaltered. Intravenous administration of 1 IU of ACTH to 4 of the dogs markedly stimulated glucocorticoid and aldosterone secretion, indicating a responsive adrenal. Intravenous administration of crude saline extracts of normal dog kidneys and partially purified renin extracts of normal and hypophysectomized dog kidneys caused a marked increase in aldosterone secretion ($p < 0.01$) within 20 minutes, and a slight to moderate increase in glucocorticoid secretion. A marked pressor response also occurred. Following the "renin" extracts, the high aldosterone secretory rates persisted through several collection periods, despite a decreasing blood pressure and a return of 17-hydroxycorticoid secretion to normal. Infusions of synthetic angiotensin II at several different rates (0.08 to 1.7 μg per minute) stimulated aldosterone secretion within 20 minutes. The smaller doses increased chiefly aldosterone secretion, while larger doses increased 17-hydroxycorticoid secretion as well. In contrast, 2 to 10 mU of ACTH stimulated 17-hydroxycorticoid secretion to equal or greater levels than the larger angiotensin doses, "renin" or crude kidney extracts, but did not stimulate aldosterone secretion. It is concluded that the kidney via the renin-angiotensin system is the mechanism by which hemorrhage stimulates aldosterone secretion in the hypophysectomized dog.

The Adrenal Glands and Calcium Metabolism. W. P. LAIRD MYERS AND WALTER LAWRENCE, JR., New York, N. Y. (introduced by Rulon W. Rawson).

In studies of antitumor agents in patients with hypercalcemia secondary to cancer, it was noted that cortisone was often effective in restoring normocalcemia. Although tumor regression seemed a reasonable explanation, patients frequently failed to show other indications of this. Therefore, the possibility of a nonspecific effect

on calcium metabolism was considered. Balance studies in 3 such hypercalcemic patients revealed no evidence that excessive absorption of calcium was responsible for the hypercalcemia. Cortisone in 1 of these patients led to an expected increase of urinary and fecal calcium without alteration of the serum calcium suggesting that the effect of cortisone on the serum calcium is independent of its effect on calcium balance. Cortisone was then found to produce reversible hypocalcemia when given to a patient with hypoparathyroidism maintained on parathyroid extract and, when given in high doses to 1 of 2 patients with functioning parathyroid carcinomas, partial reversal of hypercalcemia occurred. These data suggest a possible antagonism between cortisone and parathyroid hormone. During adrenal insufficiency in adrenalectomized dogs, hypercalcemia occurred in 12 of 19 dogs and 8 survived to show restoration of normocalcemia when cortisone was again administered. Parathyroidectomy failed to abolish this hypercalcemia of adrenal insufficiency in 7 studies in 4 dogs. Thus although an antagonism between cortisone and parathyroid hormone may exist, the hypercalcemia of adrenal insufficiency does not appear to depend upon intact parathyroids. Preliminary data in 2 dogs revealed no significant changes in serum citric acid and ultrafilterable calcium during adrenal insufficiency and hypercalcemia did not appear to be mediated through changes in serum electrolytes or plasma volume. The data thus far indicate that the adrenal glands exert a dual effect on calcium metabolism, namely, on calcium balance and on the equilibrium existing between skeletal and extracellular calcium.

Respiratory Gas Tensions and Gastric Acid Secretion. A. Naitove and S. M. Tenney,* Hanover, N. H.

The high incidence of peptic ulceration reported to be associated with chronic pulmonary emphysema has stimulated interest in the possible role that *in vivo* levels of oxygen and carbon dioxide tensions may play in modifying gastric acid secretion. Most recently, reports on emphysematous patients would seem to indicate no significant correlation between the absolute levels of either basal or histalog-stimulated acid secretion and the degree of respiratory acidosis. On normal subjects we have found that postprandial gastric acid secretion was in general directly related to the alveolar carbon dioxide tension, but for any given carbon dioxide value, secretion was inversely related to the alveolar oxygen tension. However, fasting secretion in man was not affected by altered respiratory gas tensions, and in the unanesthetized dog 7.5 per cent CO_2 caused a decrease in the level of acid secretion in a Heidenhain pouch stimulated with histalog. Clearly, oxygen and carbon dioxide can significantly modify an existing state of acid secretion, but the nature of that alteration depends on the stimulus employed for the background secretion. The interacting effects of oxygen and carbon dioxide and the possible mechanisms through which their action is exerted will be discussed.

Heme and Globin Synthesis in Maturing Human Erythroid Cells. David G. Nathan and Frank H. Gardner,* Boston, Mass.

The synthesis of heme and globin during maturation of human erythroid cells has been assessed by measurement of the daily incremental specific activity of glycine in peripheral blood hemin and globin following a single intravenous injection of glycine-2-C^{14}. These studies together with measurements of the rate of incorporation of Fe^{59} into peripheral red blood cells and hemin have been done both in adult males, patients with pernicious anemia, and a patient who received vitamin B_{12} simultaneously with the precursor radioisotopes. The results indicate that in the normal individual globin is maximally synthesized early in maturation. A linear decline in its synthetic rate occurs as the erythroid cell matures. Heme appears to be maximally synthesized midway in maturation with lower synthetic rates both early and late in erythrocyte development. In the megaloblastic maturation of pernicious anemia, heme and globin synthetic rates are closely linked, but the maturation time for many of the erythroid cells is distinctly prolonged. Vitamin B_{12} shortens the maturation time to normal and may increase the rate of globin synthesis.

Persistent Myocardial Hyperemia and Increased Oxygen Consumption During Recovery from Exercise in Man. W. A. Neill, R. J. Wagman, J. V. Messer, H. J. Levine, N. Krasnow and R. Gorlin,* Boston, Mass.

Work performed in excess of O_2 consumption (qO_2) by exercising skeletal muscle results in increased O_2 uptake during recovery. Left ventricular (LV) work also may rise more than qO_2 during effort. LV recovery metabolism, however, has not been studied. LV coronary flow (CF), qO_2, work and tension time index (TTI) were determined in 28 patients at rest, during 6 to 8 minute exercise and during a 4 minute recovery period starting either at the end of exercise or 1.5 minutes later. Exercise work and TTI increased more than CF and qO_2, and efficiency rose significantly in 50 per cent of patients. Although LV hemodynamics had returned to near rest levels, recovery CF remained significantly elevated in 71 per cent. The recovery CF level appears to be related to the degree of exercise LV stress. In 10 patients, recovery CF was much greater than at rest (+50 to +200 per cent), without high concurrent recovery LV hemodynamics. In 8 of the 10, exercise CF was comparably increased (+41 to +136 per cent); all of these had abnormally elevated LV work due to heart disease. In the remaining 2, exercise TTI was excessively increased but CF did not rise until recovery, with corresponding reciprocal changes in exercise-recovery efficiency. In 10 with smaller increases in recovery CF (average +30 per cent) exercise CF rose comparably (average +31 per cent). Recovery CF was near resting in the 8 remaining patients. Exercise CF and LV stress caused by disease or exercise were lower than in the others. Coronary O_2 extraction was constant throughout in 20 patients. In all 8 with increased extraction during

exercise, extraction remained high through recovery, suggesting persistent O_2 needs out of proportion to CF. Average efficiency was higher during exercise (+28 per cent) and lower during recovery (−16 per cent). At least part of the apparent increase in exercise efficiency may represent utilization of anaerobic or stored energy, requiring O_2 for postexercise restoration. No excess lactate production during exercise has been demonstrated.

Effect of Plasma Iodide Concentration on the Accumulation of Stable Iodide by the Human Thyroid. WIL B. NELP, HENRY N. WAGNER, JR. AND JAMES H. DOWLING, Baltimore and Bethesda, Md. (introduced by Palmer H. Futcher).

Conventional methods of measuring radioiodine uptake do not permit one to measure the rate of accumulation of stable iodide by the thyroid gland. A technique of neutron activation analysis was developed to measure quantitatively the iodine content of biological fluids. With this technique, it was possible to measure thyroidal uptake of iodide in micrograms per unit time, and to relate it to plasma inorganic iodide concentration. In 34 euthyroid subjects, the uptake of stable iodide was 6.2 μg per 2 hours (range, 1.4 to 20 μg per 2 hours). Plasma concentrations ranged from 1 to 10 μg per L. Iodide uptake was greatest when plasma concentrations were highest. To study the effect of increased plasma iodide concentrations, euthyroid subjects were given increasing amounts of oral iodide for periods as long as 47 days. The thyroid accumulated iodide at an increasing rate until the plasma iodide level reached 75 μg per L. At greater plasma concentrations, the iodide accumulation fell, but remained several times higher than normal. In euthyroid subjects subsequently given increasing amounts of dietary iodide, the radioiodine uptake (per cent dose per 2 hours) was initially 7.2 per cent when corrected for neck extrathyroidal radioactivity by the perchlorate block method. Administration of iodide sufficient to increase plasma concentrations from 5 to 50 μg per L decreased radioiodine uptake 0.1 per cent per μg per L increase in plasma concentration. At serum concentrations greater than 100 μg per L, the per cent dose uptake decreased at a slower rate. Despite the decrease in radioiodine uptake, the thyroid continued to accumulate stable iodine at a rate six times the normal value, even after oral administration of iodine for 1 month.

Levels of Plasma ACTH in Surgical and other Acutely Ill Patients. DON H. NELSON,* COLLIN E. COOPER, ERNEST M. GOLD AND E. RAY RUTHERFORD, Los Angeles, Calif.

Plasma ACTH has been measured in patients during periods of acute medical and surgical illness using the method of Lipscomb and Nelson for assay of ACTH in plasma. These were compared with simultaneous measurements of plasma 17-hydroxycorticosteroids. Levels of ACTH preoperatively have varied from nondetectable to 0.7 mu per 100 ml of plasma. One-half hour following a surgical procedure the levels have generally been between 1 and 5 mU per 100 ml. Levels at 4 hours postoperatively are usually approaching and at 24 hours have reached preoperative values. Patients who have a number of acute medical diseases have also been demonstrated to have elevated plasma ACTH. These include patients with hypoglycemia, diabetic coma, cerebral vascular thrombosis, and acute myocardial infarction. Although the very high plasma corticosteroids in terminal patients have generally been attributed to decreased metabolism of the steroid, it is of interest that such patients may also have elevated levels of plasma ACTH.

Intracellular Buffer Systems in Muscle and Brain. GEORGE NICHOLS, JR.,* Boston, Mass.

The existence of buffer systems outside the extracellular space is well known. However, little information is available concerning buffer capacities of individual tissues and the relative importance of different buffers in maintaining pH in different cells, although it seems likely from differences in function and composition that these might vary widely. To examine these possibilities, titrations of homogenate of muscle and brain freshly prepared in isotonic KCl have been performed in a closed system under a stream of nitrogen at 35° C. After measuring initial pH, aliquots of HCl were added, recording pH after each addition, until a pH of 4 or less was reached when it could be presumed that all the HCO_3^- and $CO_3^=$ initially present had been converted to CO_2 and removed by diffusion. The homogenate was then titrated back with NaOH to about pH 9. By comparing the slope of the titration curve, using acid, with the subsequent back titration, using alkali, the relative importance of the "CO_2" and the sum of the other buffer systems in each tissue could be estimated. Comparing the acid titration curves at pH 7, the total buffer capacity of brain (0.140 mEq per pH per g dry weight) was found to be slightly greater than that of muscle (0.135). However, on back titration the two values were quite different—brain buffer capacity was now only 0.081 while muscle was 0.130. It appears therefore that the CO_2 system is an important buffer in brain accounting for 42 per cent of the total buffer capacity, while in muscle this system supplies only 4 per cent and other buffers such as proteins are the important ones. The implications of these findings in terms of cell function, tissue buffering during acid-base disturbances, and the susceptibility of different organs to acidosis and alkalosis of different types will be discussed.

Micropuncture Studies of Mercurial Effect on Na Fluxes in Proximal Tubule of Necturus. DONALD E. OKEN, WILLIAM J. FLANIGAN AND RAJA N. KHURI, Boston, Mass. (introduced by C. Sidney Burwell).

Stopped flow microperfusion experiments were performed on adult Necturi anesthetized with tricaine methanesulfonate and weighing approximately 200 g. The animals received 2 mg inorganic mercury equivalent subcutaneously 24 hours prior to micropuncture. The perfusing fluid used was isosmotic with Necturus plasma,

containing 100 mmoles NaCl, 2.2 mmoles glucose, and tracer amounts of $Na^{24}Cl$ and C^{14}-inulin. Sodium efflux (tubule fluid to plasma) in 9 control experiments on 5 animals was 249 ± 27 pmoles per cm^2 second, influx 167 ± 29 pmoles per cm^2 second, and net flux 82 ± 9 pmoles per cm^2 second, values comparable with those reported previously. In 18 experiments on 11 animals receiving mercaptomerin, sodium efflux was 168 ± 11 pmoles per cm^2 second, influx 113 ± 9 pmoles per cm^2 second, and net flux 60 ± 8 pmoles per cm^2 second. Statistical analysis of the data revealed that both unidirectional fluxes and net fluxes were reduced significantly ($p < 0.01$ for influx and net flux, $p < 0.05$ for efflux). Since sodium influx into the proximal tubule is a passive process, diminution of sodium influx suggests, in the absence of a reduced transtubular electrochemical potential gradient, a decreased permeability of the tubule to sodium. Although intratubular electrical negativity is reduced after mercurials (Giebisch), this alone cannot account for the decreased sodium influx since efflux is similarly decreased. The decrease in net sodium flux observed in these experiments also is compatible with decreased tubular permeability to sodium without necessarily invoking change in the mechanisms for active sodium transport. Aldosterone, substituted for mercury at a dose of 10 μg intraperitoneally 4 to 8 hours prior to 15 similar experiments on 7 animals, failed to produce any significant change in efflux, influx, or net flux of sodium in the proximal tubule of Necturus kidney.

Metabolism of Pyruvate-2-C^{14} in Normal and Diabetic Humans. Robert E. Olson,* I. Rosabelle McManus and Paul Sweeney, Pittsburgh, Pa.

It has been postulated by some workers that a defect exists in the metabolism of acetyl-CoA via the Krebs tricarboxylic acid cycle in diabetes mellitus. In order to test this hypothesis, trace doses of pyruvate-2-C^{14} (as the sodium salt) were given intravenously to 4 fasting normal subjects and to 4 ketogenic diabetics withdrawn from insulin for 24 hours. The time course of change in total and specific radioactivities of blood pyruvate, α-ketoglutarate, lactate, glucose and respiratory CO_2 were followed for 12 hours. Pyruvate and α-ketoglutarate were isolated as their 2,4-dinitrophenylhydrazones and purified by silicic acid chromatography. The C^{14} in blood glucose was measured as CO_2 after periodate oxidation of potassium gluconate followed by Hg^{++} oxidation of carbons 2 to 5. After permanganate oxidation of the blood lactate, C^{14} in C-1 was measured as CO_2 and C^{14} in carbons 2 to 3 as acetaldehyde dimedon. The specific activity of the respiratory CO_2 reached maximal specific activity in 20 to 30 minutes in both normals and diabetics. In normals the recovery of $C^{14}O_2$ over 12 hours was 43 to 58 per cent of the administered dose, while in diabetics it was 26 to 46 per cent. The biological half-life of pyruvate in both groups was 1.2 ± 0.4 minutes. Lactate and α-ketoglutarate reached maximal specific activity equally rapidly in both groups and declined to low levels over a 2 hour period. The total C^{14} in the blood glucose was significantly elevated in the diabetic group. These findings are consistent with a diversion of pyruvate toward gluconeogenesis in the diabetic without a significant alteration in its metabolism via the tricarboxylic acid cycle.

Antidiuretic Hormone-like Effects of Adenosine-3',5'-phosphate (Cyclic AMP) and Theophylline in the Toad Bladder. Jack Orloff * and Joseph Handler, Bethesda, Md.

The response of toad bladder (*Bufo marinus*) to antidiuretic hormone (ADH) is characterized by an increase in the permeability of the membrane to water, a rise in membrane potential (P.D.) and an increase in sodium transport (short-circuit current). The effects are considered specific for neurohypophyseal principles. ADH also promotes the release of cortisol from the adrenal and glucose from liver, effects analogous to those of ACTH and epinephrine, respectively. Since the latter hormones increase cyclic AMP formation and phosphorylase activity in the specific tissues sensitive to their action, it was considered that the ADH effect in toad bladder might also involve the intermediacy of cyclic AMP. In consonance with this view, addition of 10^{-3} M cyclic AMP, but not 5' AMP, to the medium bathing the serosal surface of the toad bladder sac increases the osmotic flow of water across the membrane. In addition, P.D. and short-circuit current also rise. The effects are reversible and qualitatively indistinguishable from those produced by vasopressin (Pitressin). Theophylline (10^{-3} M), which inhibits cyclic AMP degradation in other tissues, also mimics the response to vasopressin in toad bladder. The effect of theophylline on water movement is additive to that of either a submaximal dose of vasopressin or cyclic AMP. N-ethylmaleimide prevents the changes in water movement produced by vasopressin, cyclic AMP and theophylline. Acidification of the bathing medium (pH 6.5) which inhibits the vasopressin effect, does not eliminate the response to cyclic AMP. In view of the similarity of action of vasopressin and the nonhormonal preparations studied, it is suggested that ADH may induce its effect on membrane permeability in both toad bladder and renal tubule by stimulating the formation and accumulation of cyclic AMP.

The Kinetics of Bilirubin Formation In Vivo. J. Donald Ostrow, James H. Jandl* and Rudi Schmid,* Boston, Mass.

The rate and efficiency with which hemoglobin is converted to bilirubin was studied in rats, with both hemoglobin solutions and intact erythrocytes. Small amounts of antibody-coated rat erythrocytes or free hemoglobin, labeled with C^{14} and Fe^{59}, were injected intravenously into bile fistula rats. The rate of clearance of Fe^{59} from the circulation was compared with the rate of excretion of C^{14} in bilirubin isolated in crystalline form from serial bile samples. Although biliary excretion of bilirubin-C^{14} began within 30 minutes following the injection of either

labeled erythrocytes or hemoglobin, there was a delay of 3 hours between the half-disappearance of labeled erythrocytes or hemoglobin from the circulation and half the excretion of bilirubin-C^{14}. Injection of either labeled erythrocytes or hemoglobin resulted in a temporary increase in biliary excretion of nonlabeled endogenous bilirubin. The efficiency of conversion of the injected heme-C^{14} to the excreted bilirubin-C^{14} ranged from 43 to 65 per cent. By contrast, over 90 per cent of bilirubin-C^{14} injected intravenously was recovered in the bile. These studies demonstrate that, *in vivo,* degradation of heme to bilirubin requires on the average 3 hours, although some conversion takes place within 30 minutes of sequestration of heme pigment from the circulation. The rate of conversion is similar, whether this occurs predominantly in the liver, as with free hemoglobin, or in the spleen, as with sensitized erythrocytes. In either instance, from 35 to 57 per cent of the heme moiety of hemoglobin is apparently degraded to metabolites other than bilirubin.

Bile Acid Fever and Inflammation in Man. Robert H. Palmer, Paul B. Glickman and Attallah Kappas, Chicago, Ill. (introduced by Richard L. Landau).

The pyrogenic action of neutral steroid metabolites of the 5β-H series in man has been reported previously. This study demonstrates extension of pyrogenicity to related C_{24} bile acid steroids and describes the structural and species specificity of this action. Nineteen free and conjugated bile acids were examined for thermogenic properties. Of 13 free compounds, only the 3α-monohydroxysteroid, lithocholic acid, had consistent and intense pyrogenic action in man. Hydroxylation at the 6α, 7α, 7β, and 12α positions, singly or in combination, or introduction of a ketone at carbon-7 or 7 and 12 suppressed the pyrogenicity of lithocholic acid. Derivatives of this steroid produced by C_3 acetylation, or C_{24} methylation, and the physiological glycine conjugate all had significant thermogenic and inflammatory activity. In contrast, taurolithocholic acid, another physiological conjugate, had little or no pyrogenic action but caused intense inflammation. Fever produced by lithocholic acid, like that produced by etiocholanolone and other neutral steroid pyrogens, was species specific for man. The results of this study indicate: 1) The endogenous biliary steroid, lithocholic acid, has significant inflammatory and pyrogenic action in man. Aberrations in the degradation of cholesterol to bile acids or enteric microbial dehydroxylation of these compounds may result in excessive production of this extremely active steroid pyrogen which may, by inference, then participate in febrile and inflammatory clinical disorders. 2) The potent pharmacological properties of chemical and physiological esters of lithocholic acid demonstrate that *in vivo* conjugation processes do not necessarily terminate the biological activity of steroids. 3) Production of intense inflammation without significant fever by taurine-conjugated lithocholic acid strongly suggests that nonspecific inflammation per se does not explain the mechanism of steroid fever in man.

The Mechanism of Hormonal Regulation of Glucose Oxidation in the Thyroid. Ira Pastan and James B. Field, Bethesda, Md. (introduced by J. E. Rall).

Thyroid-stimulating hormone (TSH) at a concentration of 50 μU per ml stimulates oxidation of glucose-1-C^{14} to $C^{14}O_2$ in thyroid slices. Stimulation is observed 5 minutes after addition. These observations suggest TSH acts directly on glucose oxidation in thyroid. Since the yield of CO_2 from the first carbon of glucose is greater than that from the sixth carbon, TSH appears to stimulate preferentially the hexose monophosphate pathway (HMP). During stimulation with TSH *in vitro,* there is a progressive rise in thyroidal TPN and an equivalent fall in DPN. Since TPNH and DPNH levels are unaffected, and since TPN is rate-limiting for glucose oxidation via the HMP, it is postulated that the mechanism of action of TSH is by stimulation of the conversion of DPN to TPN, a reaction carried out by DPN kinase. Pyridine nucleotide levels were measured fluorometrically and also by a new assay for TPN. In this assay which utilizes 6-phosphogluconate-1-C^{14} and 6-phosphogluconic dehydrogenase, the $C^{14}O_2$ produced is proportional to TPN. Epinephrine, adrenochrome (produced during incubation of thyroid with epinephrine), monoiodotyrosine and diiodotyrosine all increase glucose-1-C^{14} oxidation by thyroid slices. Catalytic amounts of epinephrine and adrenochrome stimulate TPNH oxidation by thyroid homogenates. Monoiodotyrosine and diiodotyrosine are deiodinated in the thyroid by a TPNH-dependent enzyme. Therefore, since a major factor in the control of glucose oxidation in the thyroid is the level of TPN, substances which alter this level alter the pathway of glucose oxidation of the thyroid.

Acquired Resistance to Allergic Encephalomyelitis and the Role of a Serum Factor. Philip Y. Paterson, S. Martin Harwin and Norman C. Didakow, New York, N. Y. (introduced by Lewis Thomas).

Allergic encephalomyelitis (AE), induced in rats by injection of nervous tissue combined with Freund's adjuvant, is under study as a model for elucidating the cellular and humoral factors implicated in autoimmune disease. Although transfer of AE was accomplished in our hands by means of lymph node cells, unsuccessful attempts to transfer it using serum leaves the role of circulating antibrain antibodies still unclear. Our recent studies indicate that rats sensitized to spinal cord and adjuvant recover promptly from AE and later exhibit a striking resistance to the disease. For example, 31 rats were permitted to recover from the paralysis induced by a single injection of spinal cord-adjuvant and then challenged 4 to 6 weeks later. Following challenge with spinal cord-adjuvant, only 1 of the 31 rats became paralyzed. Histological evidence of AE was found in this animal and in 6 others; the remaining 24 rats had no demonstrable encephalomyelopathy. As expected, 14 of 18 control rats developed AE. Serum transfer experiments indicate that a circulating factor is impli-

cated in AE resistance. Fifteen recipients were sensitized to spinal cord-adjuvant and treated on alternate days with pooled serum from donors similarly sensitized 3 weeks previously. Only 1 of these 15 recipients became paralyzed. Many AE lesions were subsequently found in this recipient as well as in 3 others. Of the remaining 11 recipients, however, 6 showed only minimal AE lesions and 5 had no evidence of AE at all. In contrast, typical AE occurred in 12 of 14 control recipients sensitized to spinal cord-adjuvant but treated with pooled serum from normal rats or donors previously sensitized to kidney-adjuvant. It is conceivable that the circulating factor which protects against the development of AE may be antibrain antibody. Studies to settle this point are in progress.

Biochemical Detection and Histological Study of Muscular Dystrophy in the Preclinical Stage. CARL M. PEARSON, SUDHIR R. CHOWDHURY AND WILLIAM M. FOWLER, Los Angeles, Calif. (introduced by William N. Valentine).

Serum enzymes are known to be elevated during the active clinically progressive stages of muscular dystrophy. Moreover, several brief reports have indicated that levels are highest early in the clinical disease and taper toward normal as weakness becomes extreme. Enzyme elevations do not occur in secondary (neuropathic) muscular weakness; hence, when present, they are indicative of a primary myopathy, if other organ disease can be excluded. We have conducted a detailed clinical and serum enzyme study of several families in which dystrophy is known to exist and have found elevated enzyme levels (glutamic oxalacetic transaminase, glutamic pyruvic transaminase, aldolase, lactic dehydrogenase, pyruvic dehydrogenase) in several young boys who were at the time without demonstrable muscular weakness. It appears that comparably high levels are present in the preclinical and early clinical stages of the disease. The youngest child was 4 months old when first tested; his enzyme levels were all very high. Four of his brothers had developed clinical dystrophy at age 3 years and it is likely that he also will. In another family, early muscular weakness developed in 3 fourth-generation offspring of a dystrophic family, 3 years *after* finding elevated serum enzymes in these children. Muscle biopsy obtained from several persons in the preclinical stage, when compared with biopsies taken in other family members with successive degrees of weakness, has shown "early" muscle fiber alterations and connective tissue increase even in the earliest patient studied (aged 4 months). Furthermore, it is apparent that much histological change, including fiber swelling, necrosis and active regeneration as well as interstitial fibrosis, occurs before clinical weakness appears, at which time the usual muscle biopsy is obtained. It is probable that both repeated muscle fiber necrosis and increased sarcolemmal membrane permeability contribute to the continuous elevation of serum enzymes. The basic biochemical defect in this disease remains to be elucidated. It seems likely that further studies of this type will provide clues as to when the dystrophic process actually begins. This will probably be in the neonatal period in some cases.

Studies with Purified Bovine Parathormone: Effects in Normal, Hypoparathyroid and Pseudohypoparathyroid Subjects. MAURICE M. PECHET,* HOWARD RASMUSSEN, EVELYN L. CARROLL AND IRMA GRAMER, Boston, Mass.

Complete metabolic balance studies were carried out with Rasmussen purified parathormone (PTH). During the first day of administration of PTH, there was a phosphorus diuresis in all subjects, especially pronounced in the normal and hypoparathyroid subjects. The phosphaturic response continued in the normal and hypoparathyroid subjects, although at a reduced level, throughout the period of PTH administration, but in the pseudohypoparathyroid subjects, the phosphorus diuresis ceased after the second to fourth day. In the normal subject after the fourth day there was a further increase in phosphorus excretion. Upon withdrawal of PTH there was a prompt, marked decrease in phosphorus excretion. In the normal and hypoparathyroid subjects the changes in calcium excretion lagged behind those in phosphorus excretion, and in the pseudohypoparathyroid subjects the changes in calcium excretion were negligible. Serum phosphorus fell and serum calcium rose during PTH administration, the changes being most pronounced in the normal and hypoparathyroid subjects. On starting PTH administration there was a prompt potassium loss for the first day or two, and on stopping PTH there was a prompt potassium retention for the first 2 days. These changes occurred *pari passu* with changes in phosphorus excretion on starting and stopping the administration of PTH. The potassium and phosphorus "on and off" effects occurred in all instances and may indicate changes in intracellular fluid. Urinary pH rose during PTH administration and then dropped sharply on the first day following withdrawal of PTH. The data can be interpreted as follows: 1) PTH induces a slow bone response; 2) PTH induces a prompt renal tubular phosphaturic response, the phosphorus coming predominantly from the intracellular fluid. The pseudohypoparathyroid subject does show a renal phosphaturic response albeit a sluggish one.

Serum Enzyme Levels Following Acute Arterial Occlusion of Dog Limbs. RAYMOND PENNEYS, UGO MANZOLI, SAMUEL BELLET AND LEONARD FEINBERG, Philadelphia, Pa. (introduced by Hugh Montgomery).

These experiments were done to relate levels of the serum enzymes, glutamic oxalacetic transaminase (GOT), isocitric dehydrogenase (ICD), and lactic dehydrogenase (LDH), to ischemia of the animal limb, in the same manner that they have been related to ischemia of the myocardium. In the first group of experiments, embolism of a hind limb was produced in 16 dogs by the injection of

a lycopodium suspension into the femoral artery. This resulted in severe, ischemic damage to the limb, progressing to gangrene in 13 of the 16 animals. All three enzymes increased within 4 hours after embolization, much before the appearance of gangrene, and remained so during the 96 to 144 hour observation period. The greatest increase was in GOT, the average value of which rose from approximately 40 U before embolization to 1,000 U, at 24 to 48 hours after embolization; the next greatest increase was in ICD, which rose from 100 to 600 U, at 12 to 24 hours; and the least was in LDH, from 100 to 300 U, at 12 to 24 hours. Sham operations on 4 dogs produced no significant rise in enzyme levels, demonstrating that embolization, per se, was responsible for the increase in serum enzymes. A second set of experiments was done to study the effect of milder degrees of ischemia of the limb. The femoral artery was occluded, by clamping, for 1 hour and for 3 hours in two groups of 6 animals each; in neither group was there any evidence of serious damage to the limb. Levels of GOT and ICD remained essentially unchanged during the 24 hour observation period after occlusion. Levels of these serum enzymes, then, are related to the degree of ischemic damage of the limb. Such enzyme changes probably reflect the degree of damage of the deeper tissues of the limb, something not provided by the routine clinical examinations, and should therefore be of value in the management of patients having acute, severe peripheral arterial obstructions.

The Nature of the Heterogeneity of Normal Adult Human Myoglobin. GERALD T. PERKOFF,* ROBERT L. HILL AND FRANK H. TYLER,* Salt Lake City, Utah.

Myoglobin has been prepared from normal human muscle obtained at autopsy. Such preparations can be separated into three major and two minor components when chromatographed at 6° C on diethylaminoethylcellulose which has been equilibrated previously with 0.005 M Tris-(hydroxymethyl) aminomethane buffer, pH 7.85, and can be separated into three components by electrophoresis on starch gels at pH 8.6. Each major chromatographic fraction migrates electrophoretically like one of the components observed on starch gels. The percentage of each major fraction (F1, F2 and F3) in whole myoglobin varies among different preparations. Generally, F1, F2 and F3 account for 55 to 65, 15 to 20 and 15 to 30 per cent, respectively, of the total myoglobin. Spectral analysis indicates that F1 is acid metmyoglobin and that F2 and F3 are alkaline metmyoglobins. F1 and F2 have identical visible and ultraviolet spectra when converted to ferromyoglobin, oxymyoglobin and cyanmetmyoglobin. The electrophoretic and chromatographic mobilities of F1 and F2 are identical when these are converted to cyanmetmyoglobin. F1 and F2 show identical peptide patterns when tryptic digests are compared by two-dimensional electrophoresis-chromatography ("fingerprinting"). Analyses of whole myoglobin and F1 by the fluorodinitrobenzene and phenylisothiocyanate end-group techniques show glycine to be the single amino terminal residue. The electrophoretic mobility of F3 cyanmetmyoglobin differs from that of F1 and F2. However, the significance of this observation is uncertain because traces of nonheme proteins present in F3 have prevented definitive characterization of this fraction. It can be concluded that F1 and F2 are identical proteins but behave differently when isolated from muscle as the result of differences in the state of the heme prosthetic group. These results must be considered in the interpretation of studies of myoglobin from patients with muscle disease.

Serum Antinuclear Factors in Patients and Relatives: Observations in SLE and Liver Disease. VICTOR E. POLLACK, ENNO MANDEMA AND MYRON GARSENSTEIN, Chicago, Ill. (introduced by Robert M. Kark).

A semiquantitative technique was developed to study antinuclear factors (ANF) in sera of patients with systemic lupus erythematosus (SLE). Fresh, unfixed smears of human buccal mucosal cells were incubated for 90 minutes with serum undiluted and in serial dilutions. After thorough washing they were incubated for 30 minutes with antihuman globulin serum conjugated with fluorescein isothiocyanate and examined microscopically with ultraviolet light. The "titer" of ANF was the highest serum dilution with which specific nuclear fluorescence was demonstrated. Appropriate immunologic controls were done, and reproducibility was established. The demonstration of specific nuclear fluorescence with serum diluted 1:4 distinguished clearly between SLE patients and healthy subjects, and the demonstration of specific nuclear fluorescence with serum diluted $\geq 1:4$ was called a positive test. Negative tests were found in all 40 healthy subjects, and in 71 of 75 patients with diseases "unrelated to SLE." By contrast, 75 of 81 SLE patients had one or more positive tests; in all, positive tests were found with 225 of 248 sera from SLE patients and ANF titers up to 1:4,096 were recorded. ANF were found in sera of 18 of 82 patients with disorders "related to SLE"; e.g., scleroderma, rheumatoid arthritis. ANF were studied in sera of 125 relatives of 21 SLE patients; most were asymptomatic, but three new cases of SLE were found. Positive tests were found, many in high titer, in 44 of 85 first degree and in 9 of 40 second degree relatives, indicating that in SLE a tendency to develop antinuclear factors is inherited. Preliminary control studies on families of healthy subjects were negative. ANF were also demonstrated in one acute and 4 chronic cases of hepatic disease with hypergammaglobulinemia. To date two families have been studied; ANF were demonstrated in 6 of 9 relatives, suggesting a relationship between SLE and "lupoid hepatitis."

Presence of an Erythropoietic Labile Iron Pool. MYRON POLLYCOVE, Berkeley, Calif. (introduced by John H. Lawrence).

The concept of an erythropoietic labile iron pool is central to models previously developed and employed to

calculate hemoglobin synthesis, mean erythron life span, storage iron deposition and miscible storage iron in over 480 human subjects. This pool is much more labile than miscible storage iron which corresponds in amount to ferritin and is calculated to be 85 mg in normal subjects (heme iron in immature erythrons, approximately 30 mg). To investigate the presence of a corresponding morphologic or biochemical entity, human and canine marrow and circulating immature erythrons were analyzed directly. Within 2 hours after intravenous injection of tracer amounts of transferrin-bound radioiron, Fe^{59} was concentrated chiefly in marrow cells. Doubly crystallized heme was prepared from marrow and circulating immature erythrons. Soon after injection, radioiron occurs chiefly in nonheme form which decreases exponentially as heme radioiron increases. Approximately half of the radioiron was present in nonheme form at 36 hours in man and 7 hours in dog, in close agreement with rates calculated from simultaneous iron kinetic studies. Erythron lysis and isolation of membranes demonstrated that nonheme radioiron was on the membrane, while heme radioiron was present almost entirely in the interior. Fe^{59}-labeled reticulocytes were obtained from patients 30 to 60 minutes after intravenous injection of Fe^{59}. After incubation for 1 hour in plasma, 12 to 18 per cent of the reticulocyte radioiron transferred to plasma transferrin in proportion to plasma iron-binding capacity; less than 2 per cent transferred to the control ACD solution. In conclusion: 1) A labile iron pool is present in immature erythrons. 2) This pool is predominantly on the cell membrane, derives iron from plasma transferrin, transfers iron back to plasma transferrin, and furnishes iron to the interior for heme synthesis. 3) Iron in excess of heme iron is removed by feedback to plasma.

Renal Effects and Site of Action of Organomercurials in Hydropenic Man. JEROME G. PORUSH, MARVIN H. GOLDSTEIN, GILBERT M. EISNER AND MARVIN F. LEVITT,* New York, N. Y.

Previous studies in this laboratory demonstrating that free water clearance remained constant during a mercurial diuresis in hydrated man suggested that the major site of organomercurial action is distal to the water-clearing segment. To test this hypothesis in hydropenic man, the characteristics of the urine (V, C_{osm}, and $T^c_{H_2O}$) were studied during comparable diureses produced by nonspecific solute diuretics (aminophylline, hypertonic salt and mannitol) and organomercurials (meralluride, mersalyl and mercaptomerin). With the nonspecific agents, as C_{osm} increased from 1.5 to 15 ml per minute, a progressive rise in $T^c_{H_2O}$ was observed to peak values of 6.6, 6.9, and 5.4 ml per minute for aminophylline, salt, and mannitol, respectively. During comparable organomercurial diureses, $T^c_{H_2O}$ remained constant at those levels which prevailed prior to the onset of the saluresis, despite a wide range in control $T^c_{H_2O}$ values (1.0 to 7.0 ml per minute). In 10 of 12 experiments in which a nonspecific agent was superimposed during a mercurial diuresis, $T^c_{H_2O}$ increased an average of 2 ml per minute. In other studies, blood pressure was abruptly reduced during comparable diureses produced by organomercurials or hypertonic salt infusions. The subsequent fall in glomerular filtration rate was associated with a comparable fall in C_{osm} in both groups, but only those subjects receiving salt displayed an appreciable increase in urine osmolality. Urine osmolality was virtually unchanged in the mercurialized subjects during the hypotension. These findings suggest a disparity in solute transport by the loop of Henle, at every level of solute excretion, between a mercurial and a nonspecific solute diuresis. The capacity to maintain or increase $T^c_{H_2O}$ during a mercurial diuresis argues against a block at this site. The relatively diminished rate of loop transport in the mercurial experiments therefore supports the hypothesis that the major effect of organomercurials occurs distal to the loop of Henle.

Dependence of Bile Formation on Bile Salt Secretion in the Dog. RUDOLF PREISIG, HENRY O. WHEELER * AND HERBERT L. COOPER, New York, N. Y.

Bile flow and electrolyte excretion were measured during rates of bile salt secretion ranging from 6 to 125 μmoles per minute (maintained by intravenous infusion of 1.3 per cent sodium taurocholate) in 40 studies on 4 cholecystectomized, unanesthetized dogs (20 to 25 kg) with Thomas duodenal cannulae. Bile flow (0.04 to 1.02 ml per minute)—during cholinergic blockade with intravenous pipenzolate methylbromide, 0.4 mg per minute—was directly proportional to taurocholate secretion rate in each dog. The proportionality factors were 0.007, 0.007, 0.009 and 0.010 ml bile per μmole taurocholate secreted. Excretion rates of chloride and bicarbonate were also approximately proportional to taurocholate secretion rate over the entire range in two dogs and at secretion rates above 20 μmoles per minute in the other two. Proportionality factors were 0.37, 0.33, 0.58 and 0.62 μEq chloride and 0.15, 0.11, 0.27 and 0.20 μEq bicarbonate per μmole taurocholate secreted. During intravenous secretin (2.5 U per minute) sufficient to produce maximal choleretic response, bile output was augmented in each dog by an approximately constant amount (0.28 0.29, 0.22 and 0.33 ml per minute) regardless of taurocholate secretion rate. Secretin-induced increments in chloride (28, 31, 29 and 47 μEq per minute) and bicarbonate (24, 33, 18 and 26 μEq per minute) were also approximately constant and independent of taurocholate secretion. Pipenzolate methylbromide did not depress the choleretic response to intravenous secretin, or to endogenous secretin elaborated during intraduodenal 0.05 N HCl administration. Thus the excretion of biliary water, chloride and bicarbonate apparently depends upon secretion of bile salts. This phenomenon is consistent with the view that water and inorganic electrolytes enter the bile along electrochemical gradients created by active bile salt secretion. Secretin-induced choleresis appears to involve an additional and altogether independent mechanism.

The Role of α-Globulins as Insulin-Carrying Proteins in Human Serum. THADDEUS E. PROUT, VASANT V. ODAK AND GEORGE J. DENDRINOS, Baltimore, Md. (introduced by Samuel P. Asper, Jr.).

Electrophoretic migration of radioinsulin (I^{131}-insulin), either alone or in serum and in various supporting media, is similar to that of α_1-globulin. Thus, it has not been possible to determine whether insulin added to serum remains free or is bound to protein. Electrophoresis on agar gel offers distinct advantages over other media for the study of this problem: *a*) albumin and α-globulins migrate toward the anode while β- and γ-globulins are separated by endosmotic flow in the direction of the cathode; and *b*) insulin alone moves to a position well ahead of the fastest moving albumin fraction, whereas insulin added to serum migrates with α-globulins. Upon addition of large quantities of insulin to normal serum (10 U per ml), insulin migrates not only with the α-globulins but also into the area ahead of albumin. Thus, saturation of binding proteins allows further migration of the excess free insulin. Serum, containing 50 mU I^{131}-insulin per ml, was partitioned by agar electrophoresis and challenged with antihuman rabbit serum by diffusion from a lateral reservoir. Radioautography demonstrated the presence of radioactivity in the precipitin arcs formed by the α-globulins. These observations provide evidence that insulin in normal serum is bound to the α-globulins. The capacity of α-globulin to bind insulin appears to be approximately 10 mU per ml of normal serum. Insulin resistance in some diabetic patients is related to an acquired insulin-binding γ-globulin. The γ-globulin in serum of an insulin-resistant diabetic was capable of binding approximately 300 mU of insulin per ml and removed insulin from the carrying protein of normal serum. The affinity for insulin by the acquired insulin-binding globulin of resistant patients is far greater than that of their insulin-carrying proteins. This suggests that the presence of insulin on the normal carrying protein is necessary for clinical control of diabetes.

Direct Effects of Ethylenediamine Tetraacetate on Renal Excretory Function. THEODORE N. PULLMAN,* A. R. LAVENDER AND IMPI AHO, Chicago, Ill.

Ethylenediamine tetraacetate (EDTA) binds polyvalent metal ions in a ring complex thereby inactivating the chelated ion. To maximize renal effects of EDTA while minimizing its systemic effects we have infused it slowly in dilute solution directly into one renal artery in the dog. The experiments were performed on fasting hydropenic anaesthetized animals. A renal artery was cannulated and normal saline slowly infused into it. The ureters were separately catheterized. After several control periods EDTA was added to the arterial infusate. All animals showed prompt predominantly unilateral natriuresis, chloruresis, and diuresis. Differential sodium excretion, negligible during pre-EDTA periods, increased to 100 to 200 μEq per minute during EDTA administration. Much smaller increases in potassium excretion also occurred. The natriuresis was not accountable for by small initial increases in filtration rate. EDTA also induced markedly increased differential calcium excretion which tended to parallel EDTA excretion mole for mole. Intrarenal infusion of calcium-EDTA chelate (CaEDTA) produced similar effects of much smaller magnitude, in some instances almost negligible. The effects of CaEDTA on calcium and EDTA excretion, however, were indistinguishable from those occurring with free ligand (EDTA). Preliminary experiments with ZnEDTA (of higher stability than CaEDTA, zinc being bound preferentially to calcium) indicate that this chelate produces slight natriuresis and negligible calciuresis but with EDTA excretions as high as with free ligand. MgEDTA effects (lower stability constant than CaEDTA) resembled those due to free ligand. These data establish that EDTA exerts a saluretic and diuretic effect by an action directly on the renal tubule. We have not established a mechanism for these effects, but suggest the possibility that it may involve chelation of an ion critical to the operation of a sodium transport system.

Pyrimidine Biosynthesis in Man: Suppression In Vivo by Anti-neoplastic Agents. MITCHELL RABKIN, ELIZABETH FREDERICK, MYRON LOTZ AND LLOYD H. SMITH, JR.,* Boston, Mass.

Many *in vivo* studies have been carried out on purine metabolism in man because of the presence of a readily available end-product, uric acid. In the absence of a comparable unique end-product, no previous over-all measurements of pyrimidine metabolism in the intact organism have been possible. In the biosynthetic sequence of pyrimidine nucleotide formation, an irreversible decarboxylation occurs in the conversion of orotidine-5′-phosphate to uridine-5′-phosphate. The release of CO_2-C^{14} from carboxyl-labeled pyrimidine precursors should therefore reflect *in vivo* pyrimidine nucleotide formation. Experiments were designed to test this hypothesis and the usefulness of this procedure in studying pyrimidine biosynthesis *in vivo* and its suppression by selected anti-neoplastic agents. Following intravenous administration of carboxyl-labeled orotic acid in the rat, approximately 50 per cent of the isotope appeared as respiratory CO_2-C^{14} within 2 hours. An additional 35 per cent appeared as unaltered urinary orotic acid. In sharp contrast, ring-labeled orotic acid released minimal CO_2-C^{14}, probably reflecting nucleotide or nucleic acid catabolism. The administration of carbamylaspartic acid, the earliest unique pyrimidine precursor, demonstrated a similar dichotomy in rate of CO_2-C^{14} evolution from carboxyl- and noncarboxyl-labeled substrates. Studies with competitive acceptors of 5-phosphoribosylpyrophosphate suggested that the availability of this reactant was not limiting. In the rat, administration of 6-azauridine, 6-azauracil, or 5-fluoro-orotic acid markedly inhibited pyrimidine nucleotide synthesis, as indicated by the diminished appearance of respiratory CO_2-C^{14} from carboxyl-labeled orotic acid. Corresponding studies in man have

demonstrated the usefulness of this procedure in estimating over-all pyrimidine biosynthesis. In patients receiving certain anti-neoplastic agents, it has allowed an *in vivo* measurement of the degree of suppression of this metabolic sequence.

The Alveolar Diffusing Volume and the Water Space of the Pericapillary Tissues of the Lungs as Measured by Multiple Indicator Dilution Curves. LLOYD RAMSEY, W. PUCKETT, A. JOSE AND W. LACY, Nashville, Tenn. (introduced by C. R. Park).

When a solution containing albumin-I^{131}, tritiated water, and nitrous oxide is injected into the right atrium, each substance will be distributed into a different physiological compartment during its first transit through the pulmonary capillaries. Albumin is confined to the vascular compartment; tritiated water mixes in an additional pericapillary tissue space; the nitrous oxide compartment includes the tritiated water space plus the alveolar gas volume which is available to the pulmonary capillaries for gaseous diffusion. Blood samples are collected anaerobically from a systemic artery at 1-second intervals following simultaneous injection of the three indicators. Albumin-I^{131} and tritiated water in each sample are determined by scintillation counting, and nitrous oxide by gas chromatography. Since flow is the same for all three indicators, the difference in shape of the three dilution curves is caused by the different volumes into which the indicators distribute. The difference between the distribution volumes of tritiated water and nitrous oxide (corrected for the partition coefficient of nitrous oxide between pulmonary tissue and air) is the alveolar diffusing volume. The difference between albumin-I^{131} and tritiated water volumes (corrected for the ratio of water content in whole blood and pulmonary tissue) is the pericapillary tissue volume of the lung. The alveolar diffusing volume and pericapillary tissue volume in dogs are 17 and 3 ml per kg, respectively, whether calculated by mean transit time or slope volume differences. The values appear to be smaller in humans. Patients with mitral valve abnormalities showed a twofold elevation of pericapillary tissue volume at rest which increased with exercise. Experimental pulmonary edema in dogs caused a fourfold increase in this measurement. Unilateral pneumothorax caused striking decrease in alveolar diffusing volume without change in pericapillary tissue volume.

Urinary Excretion of Calcium in Wilson's Disease (Hepatico-lenticular Degeneration). RAYMOND V. RANDALL, NORMAN P. GOLDSTEIN, JOHN B. GROSS, JOHN W. ROSEVEAR AND WARREN F. MCGUCKIN, Rochester, Minn. (introduced by Randall G. Sprague).

It has been reported that some patients with Wilson's disease have excreted excessive amounts of calcium in the urine and have had renal stones or nephrocalcinosis. Since it is not known whether the hypercalciuria represents another of the renal tubular defects associated with this disease, we have investigated the urinary excretion of calcium in each of 3 patients with Wilson's disease while varying widely the dietary intake of calcium. The first patient's urinary excretion of calcium averaged 106, 335, and 413 mg per day with a daily intake of calcium measuring 120, 1,538, and 2,352 mg, respectively. The second patient's urinary calcium averaged 121, 299, 348, and 475 mg per 24 hours with a daily intake of 100, 648, 1,229, and 2,347 mg of calcium, respectively. The third patient's urinary values for calcium averaged 238, 468, and 629 mg on intakes of 113, 592, and 1,620 mg of calcium, respectively. With each change in the dietary intake of calcium there was an equally abrupt change in the urinary excretion of calcium. Other investigators have shown repeatedly that as the intake of calcium varies there is relatively little change in the urinary excretion of calcium by normal persons. By contrast, our patients with Wilson's disease showed a three- to fourfold increase in the urinary excretion of calcium as the intake increased. The range as well as the abruptness of the change in the urinary excretion of calcium suggests that this phenomenon primarily reflects increased intestinal absorption. The relationship of this to the increased absorption of copper from the intestine in Wilson's disease is not known.

The Activation of Christmas Factor by Activated Plasma Thromboplastin Antecedent. OSCAR D. RATNOFF,* Cleveland, Ohio.

This study confirms the hypothesis that plasma thromboplastin antecedent (PTA), activated "spontaneously" or by glass-activated Hageman factor, converts Christmas factor from an inactive to an active form. This study demonstrates, then, that the initial steps in clotting are the successive activations of Hageman factor, PTA and Christmas factor. Previously, Biggs and Bidwell found that only after a preliminary incubation period would mixtures of PTA-deficient and Christmas factor-deficient serums behave like normal serum in the thromboplastin generation test, supporting this view. Christmas factor-like activity was measured specifically, using the partial thromboplastin time. Platelet-deficient plasma, deficient in antihemophilic factor, was recalcified and incubated in glass. At intervals, aliquots were assayed for Christmas factor. An increase of four- to eightfold in Christmas factor activity occurred within 6 minutes. Thus, antihemophilic factor was not needed for the activation of Christmas factor. Similarly, Christmas factor activity evolved in plasmas deficient in pro-SPCA (factor VII), Stuart factor or proaccelerin, but not in plasmas deficient in Hageman factor, PTA or Christmas factor. However, Christmas factor activity evolved normally in PTA-deficient plasma in which the defect was corrected by adding Hageman factor-deficient plasma (containing PTA) or crude activated PTA. Pro-SPCA-deficient plasma which had not been in contact with glass was adsorbed with aluminum hydroxide gel. An eluate of this gel, containing Christmas factor, Stuart factor and prothrombin, was incubated with

partially purified, activated PTA and calcium. Again, Christmas factor activity increased. Thus, the apparent activation of Christmas factor by activated PTA occurred in plasma or fractions deficient in pro-SPCA, Stuart factor, antihemophilic factor or proaccelerin. It required calcium and proceeded more rapidly at 37° than at 1° C. Neither platelets nor glass-like surface were required. Whether unrecognized intermediate steps are needed to activate Christmas factor is as yet undetermined.

The Mechanism of Renal Chloride Reabsorption. FLOYD C. RECTOR, JR. AND JAMES R. CLAPP, Dallas, Tex. (introduced by Donald W. Seldin).

Renal reabsorption of Cl is thought to be a passive process driven by the electromotive forces generated by active Na reabsorption. The lowest distal tubular Cl concentration ($[Cl]_L$) achievable by passive reabsorption would be determined by the transtubular potential difference (E_T), according to the Nernst equation—$E_T = -61.5 \log \text{plasma } [Cl]_P / [Cl]_L$. To determine whether Cl reabsorption in the distal tubule is an active or a passive process, the relationship between the $[Cl]_P/[Cl]_L$ ratio and distal E_T was studied in normal and Na-depleted rats during Na_2SO_4 diuresis utilizing micropuncture techniques. E_T was measured with glass microelectrodes filled with 3.0 M KCl; [Cl] in plasma and distal tubular fluid was determined with a Ramsay microchloride apparatus, type II. Mean distal E_T was −60 mv (lumen negative) in normal rats and −71 mv in Na-depleted rats. The highest E_T was −120 mv (in Na-depleted rat). The average $[Cl]_P$ was 100 mEq per L. Therefore, if Cl reabsorption were purely passive, the lowest $[Cl]_L$ attainable from the highest measured E_T would be 1.0 mEq per L. The $[Cl]_L$ was measured in 221 instances. Of these, 24 fell below the critical level of 1.0 mEq per L; 8 were between 0.5 and 1.0 mEq per L, 11 were between 0.1 and 0.5 mEq per L and 5 were between 0.01 and 0.1 mEq per L. To reduce $[Cl]_L$ to the lowest measured levels (< 0.1 mEq per L) by a passive process would require E_T ranging from −180 to −240 mv, values considerably higher than any observed E_T. It is concluded, therefore, that Cl is being reabsorbed in the distal tubule against its electrochemical gradient by a process of active ion transport.

Relation of Myocardial Carbohydrate Metabolism and Ion Transport to Contractility. TIMOTHY J. REGAN, PATRICK H. LEHAN, MARTIN J. FRANK, ERNEST N. LANDY AND HARPER K. KELLEMS,* Jersey City, N. J.

Enhanced glucose uptake and metabolism have been postulated as important to the activity of digitalis. In the intact anesthetized dog glucagon-free insulin, 0.2 U per kg, has been used to examine the time course of its effects upon myocardial glucose uptake and metabolism, and net potassium transfer. Direct injection through a left intracoronary catheter minimized changes in arterial glucose, K^+, and catechols. Sequential arteriovenous differences have been employed as a measure of substrate extraction, while the duration of isometric contraction and maximal rate of rise in left ventricular pressure served as indices of myocardial contractility. In 8 animals the maximal insulin effect on myocardial glucose and K^+ uptake, and upon the respiratory quotient occurred within 30 minutes. During this time no change in contractility was observed, implying that the digitalis influence on carbohydrate metabolism is unrelated to its inotropic activity. Although a 25 per cent increment in extracellular potassium during strophanthidin administration does not inhibit the myocardial contractile response, or the net K^+ loss elicited by the drug, insulin's ability to induce uptake of K^+ may alter the response if the ionic egress contributes to inotropism. Strophanthidin, 0.03 mg per kg, was given systemically to 8 animals during the period of insulin's metabolic activity in the myocardium. In each instance the contractile response was prevented or minimized by insulin, when compared to prior or subsequent strophanthidin response in the same animal. While this suggests a critical role of ion transport in strophanthidin's activity, the alternative explanation that insulin competes for common binding sites may be more valid. In preliminary data, *p*-chloromercuribenzoate, given in place of insulin, was found to equally inhibit the contractile response to strophanthidin. In conclusion: 1) the effects of insulin upon glucose transport and oxidation did not change myocardial contractility, so that these effects appear unlikely as essential to digitalis activity. 2) Intracoronary insulin prevents the inotropic response to strophanthidin by interfering with potassium transfer or competing for common binding sites. The latter is favored since *p*-chloromercuribenzoate exerts a similar inhibiting effect.

Effects of a Side-to-Side Portacaval Shunt on Hepatic Hemodynamics. TELFER B. REYNOLDS,* ALLAN G. REDEKER, HARRY S. YAMAHIRO AND WILLIAM P. MIKKELSEN, Los Angeles, Calif.

Pre- and postoperative evaluation of hepatic hemodynamics by hepatic vein catheterization has been performed in 9 cirrhotic patients undergoing side-to-side portacaval shunt for esophageal varices. Preoperative hepatic blood flow (HBF) ranged from 565 to 2,405 ml per minute. There was a postoperative fall in every case, averaging 59 per cent in the group as a whole. Wedged hepatic vein pressure fell 55 per cent (average) and post-sinusoidal hepatic vascular resistance did not change significantly. Splanchnic oxygen consumption fell 37 per cent in spite of consistent increases in arterial-hepatic vein oxygen difference. The fall in HBF after side-to-side shunt was significantly greater than after end-to-side shunt in a comparable group of patients studied previously. This suggests retrograde flow of hepatic arterial blood in the portal vein through the side-to-side shunt into the vena cava. Theoretically, this blood flowing in a retrograde fashion could be

perfusing hepatic tissue functionally. Direct sampling from the hepatic limb of the portal vein at the time of side-to-side shunt suggests that the volume of retrograde flow can vary from 150 to 800 ml per minute, that moderate extraction of oxygen from this blood by the liver may occur, but that sulfobromophthalein extraction is limited. Our data thus far indicate that an end-to-side portacaval shunt is less disturbing to the physiology of the liver than is a side-to-side shunt.

Pneumonia and Hemorrhagic Otitis Media in Volunteers Infected with Eaton Agent. DAVID RIFKIND, ROBERT M. CHANOCK,* HOWARD M. KRAVETZ, KARL M. JOHNSON AND VERNON KNIGHT,* Bethesda, Md.

Second monkey kidney culture passage Eaton agent, originally derived from a patient with primary atypical pneumonia, was given to 34 adult volunteers, 17 without measurable fluorescent stainable antibody and 17 with such antibody. Serologic studies indicated that all of the former group and 13 of the latter group became infected. Eaton agent was recovered in tissue culture from 9 of the 16 volunteers tested. The agent was detected in the throat for varying intervals from the fourth to the tenth day following inoculation of 64 egg infectious doses. Eight of the 17 volunteers without antibody developed illness with objective physical findings; 3 had pneumonia and 6 had hemorrhagic otitis media (one volunteer developed both otitis and pneumonia). Of the remaining 9 volunteers in this group 3 developed minor upper respiratory illness while 6 did not become ill. Otitis developed in one of the volunteers who possessed Eaton antibody prior to challenge; there was no pneumonia, 4 developed mild upper respiratory disease and 12 remained well. Aggregation of illness with physical findings (pneumonia and/or otitis) in the antibody negative group is evidence that such illness was induced by the Eaton agent. The incubation period for mild respiratory illness, otitis, and pneumonia averaged 7, 8, and 11 days, respectively. Nine of 17 without prior antibody developed cold agglutinins while none of 17 in the antibody positive group developed such agglutinins. While streptococcus MG agglutinins developed in 1 patient with pneumonia and in 1 with otitis, there was no correlation between the carrier state for this organism and the development of clinical illness. These findings corroborate a previous observation of association of Eaton agent with naturally occurring atypical pneumonia and additionally suggests that hemorrhagic, nonsuppurative otitis media may be caused by the same agent.

The Determination of Intracellular pH in Normal Human Subjects. EUGENE D. ROBIN,* PHILIP A. BROMBERG AND ROBERT J. WILSON, Pittsburgh, Pa.

There is increasing evidence that under a variety of circumstances, intracellular pH may be independent of extracellular pH. For this reason, a technique for the measurement of intracellular pH in man would be of considerable theoretical and practical interest. Following the suggestion of Waddell and Butler, such a technique has been developed, using the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), as a pH indicator. From the mass action law it may be shown that $(H^+)i/(H^+)e = (DMO^-)e/(DMO^-)i$. From simultaneous measurements of plasma pH, plasma total DMO concentration, extracellular water (sucrose space), and total body water (antipyrine space), "mean" whole body intracellular pH can be calculated. Such measurements have been performed on 9 normal men. Whole body intracellular pH averaged 6.94 ± 0.09 U at a time when arterial plasma pH averaged 7.44 U and arterial CO_2 tension averaged 31 mm Hg. The reproducibility of individual determinations was 0.04 pH units. Apparent "mean" intracellular HCO_3^- concentration averaged 7.90 ± 1.10 mmoles per L water. Parallel studies have been performed in the dog under control conditions, and following CO_2, $NaHCO_3$, "Tris" buffer, and HCl administration. These studies are consistent with rapid intracellular equilibration of CO_2 and Tris, and relatively slow equilibration of H^+ and HCO_3^-. The use of this technique should be of value in clarifying the disturbances of acid-base balance in various clinical disorders.

Depletion of Thyroid Iodine Induced by Thyrotropin in the Rat. I. N. ROSENBERG* AND C. Y. SASSON, Boston, Mass.

The thyroid content of iodinated amino acids was studied in individual rats before and shortly after thyrotropin (TSH) injection. Rats, which 24 hours earlier had received tracer I^{131}, were given propylthiouracil (10 mg) subcutaneously to minimize further organic binding of iodine; 30 minutes later, right thyroid lobectomy was done (ether), TSH (3 USP U) was then injected intravenously, and 3 hours later the left lobe of the thyroid was removed. After determination of I^{131} concentration, each lobe was digested (pancreatin), and the fraction of the radioactivity present as iodothyronines (T_X), diiodotyrosine (DIT) and monoiodotyrosine (MIT) was determined by quantitive paper chromatography (butanol : acetic acid). In 18 control experiments, only small differences were recorded between right and left lobes in I^{131} concentration and in the relative proportions of the labeled amino acids. In 20 TSH-injected animals, although the average I^{131} concentration of the left lobe was 43 per cent less than that of the right, the proportions of the labeled T_X, DIT and MIT were essentially the same in both lobes. The average concentrations of labeled T_X, DIT and MIT were, respectively, 45, 43 and 43 per cent lower in the left lobe than in the right. From these data and the specific activity of selected portions of the chromatograms (13 rats) the average losses of iodine as T_X, DIT and MIT were calculated to be, respectively, 0.034, 0.065 and 0.045 μg per mg thyroid in the post-TSH period. Specific activity considerations and the propylthiouracil block make it unlikely that disappearance of iodotyrosines could be accounted for by conversion to iodothyronines. The results suggest that under these

experimental conditions a considerable fraction of the glandular iodine released as a consequence of TSH injection may be nonhormonal.

Extra-renal Production of Factor(s) Stimulating Erythropoiesis. WENDELL F. ROSSE AND THOMAS A. WALDMANN, Bethesda, Md. (introduced by Nathaniel I. Berlin).

This investigation sought to determine whether the kidney is the sole source of erythropoietic-stimulating factor(s) (erythropoietin). Forty pairs of parabiotic rats were divided into 4 groups of 10 pairs each and nephrectomy or ureter ligation was performed on 1 of the partners of each pair. They were starved and placed in divided chambers for 16-hour periods on 2 successive days so that one partner could receive a hypoxic gas mixture (9 per cent O_2) and the other, room air at 2 L per minute. Erythropoiesis was measured by determining the per cent incorporation of Fe^{59} into the circulating red blood cells 16 hours following the intraperitoneal injection of the isotope into each of the parabiotic partners. The radioactivity per ml of blood was the same in each partner of a pair. The groups used and the results of Fe^{59} incorporation follow. Group I: anoxia to neither partner, one partner nephrectomized, 7.5 per cent; Group II: anoxia to the nephrectomized partner, 12.5 per cent; Group III: anoxia to the non-nephrectomized partner, 19.7 per cent; Group IV: anoxia to the ureter-ligated partner, 20.2 per cent. The significant differences between Groups II and III ($t \pm 2.53$, p 0.02) and between Groups II and IV ($t \pm 2.17$, p 0.05) indicate that the kidney is an important source of factor(s) controlling erythropoietic response to hypoxia. However, the highly significant difference between Groups II and I ($t \pm 2.98$, $p < 0.01$) suggests that the kidney is not the sole source of this factor(s).

Systemic and Coronary Hemodynamic Effects of an Amine Oxidase Inhibitor (J. B. 516). G. G. ROWE, S. ALFONSO, C. J. CHELIUS, H. P. GURTNER AND C. W. CRUMPTON,* Madison, Wis.

An amine oxidase inhibitor, J. B. 516 (Lakeside Lab.) was tested for its systemic (Fick) and coronary hemodynamic (N_2O method) effects in a series of intact anesthetized mongrel dogs. The compound had no discernible hemodynamic effects. Therefore a series of animals pretreated with J. B. 516 was given serotonin by continuous infusion and systemic and coronary hemodynamic effects measured again. Except for slightly greater tachycardia and somewhat more hypotension in the systemic and pulmonary arteries and in the right atrium, this series showed the same general qualitative and quantitative hemodynamic effects of serotonin as did a series of animals which had not been pretreated with J. B. 516 (i.e., pulmonary arterial pressure rose, coronary vascular resistance fell and coronary blood flow increased with narrowing of the arteriocoronary sinus oxygen difference). The effect of serotonin on J. B. 516-pretreated dogs was also similar to that of serotonin alone when administered into the left ventricle or left atrium of another series of animals except that when it was given into the left side of the heart, the pulmonary arterial pressure did not rise and right atrial pressure decreased more. The series of experiments indicates that under the conditions of these experiments serotonin is not inactivated in the lung as far as its hemodynamic effects are concerned. Administration of J. B. 516 does not change significantly the hemodynamic effects of a continuous infusion of serotonin.

Response of Bronchial Blood Flow to Tissue Proliferation in the Human Lung. THOMAS J. RYAN AND WALTER H. ABELMANN,* Boston, Mass.

If pathologic tissue proliferation requires an increased regional blood flow, proliferative pulmonary disease should be accompanied by an increased bronchial flow. Bronchial flow was measured in 37 patients as left ventricular output ($\dot{Q}_{LV}$) minus right ventricular output ($\dot{Q}_{RV}$) using indicator dilution, injected into the superior vena cava and sampled simultaneously from pulmonary and brachial arteries. Differences are expressed as per cent $\dot{Q}_{LV}$. In 19 control patients without intrinsic lung disease, $\dot{Q}_{RV}$ exceeded $\dot{Q}_{LV}$ by a mean of 6.6 per cent (SD 5.7). In 12 patients with actively proliferating lung disease, $\dot{Q}_{LV}$ was uniformly greater than $\dot{Q}_{RV}$ by a mean value of 10.5 per cent (SD 7.2), which differs significantly from the control group ($p < 0.01$). Pulmonary disease studied included 6 proven primary carcinoma, 2 proven granulomatous disease, 1 lobar pneumonia and 1 lung abscess. The increment of bronchial flow was related to the extent of pulmonary tissue involved in neoplasia as well as the stage of healing in lung abscess. In contrast, 4 patients with pulmonary emphysema or fibrosis, representing chronic lung disease without active tissue proliferation, resembled the control group: $\dot{Q}_{RV}$ uniformly exceeded $\dot{Q}_{LV}$ by a mean value of 9.7 per cent. A subsequent modification of the method involved the injection of two different indicators, separately and in rapid succession, into right and left atria for the inscription of dye dilution curves from the pulmonary and brachial arteries, respectively. In 7 determinations on control subjects, $\dot{Q}_{LV}$ uniformly exceeded $\dot{Q}_{RV}$ by a mean value of 4.0 per cent (range, 0.9 to 7.5 per cent). The present method, while not requiring left heart catheterization, leads to an underestimation of $\dot{Q}_{LV}$ and, hence, of bronchial flow. This is attributed in part to the undetected early recirculation of indicator. The data indicate that active tissue proliferation within the human lung may be associated with a substantial increase in bronchial blood flow.

Reduction in Postprostatectomy Bleeding by ε-Aminocaproic Acid. EDGARDO SACK, THEODORE H. SPAET,* RALPH L. GENTILE AND PERRY B. HUDSON, New York, N. Y.

ε-Aminocaproic acid (EACA) is an inhibitor of plasminogen activation and effective against urokinase.

Its application as a hemostatic agent following prostatic surgery was first suggested by the studies of McNicol and co-workers. Unselected patients undergoing prostatectomy were studied by the double blind method. Either EACA or saline placebo was given in identical manner in random order. EACA was given at the rate of about 5 g every 6 hours by intravenous infusion, and was continued from completion of surgery for a period of about 60 hours. This dose was selected because preliminary studies in normal subjects demonstrated that less gave unreliable urokinase depression. During this period the total urine output was collected for urokinase and hemoglobin determinations. Effective suppression of urokinase activity was achieved in all patients given EACA. Little difference in hemoglobin output between the experimental and control groups was seen in the first 12 hours after operation, but thereafter the differences were striking. The total hemoglobin output was appreciably less in the EACA-treated group and the difference from the control group was highly significant ($p < 0.0001$). The average hemoglobin loss in the control group was about eightfold that of the treated. No evident EACA toxicity was found. An interesting by-product of the study was that perineal prostatectomy was an almost bloodless operation, whereas surgery by the suprapubic route led to considerable blood loss. The effects of EACA were most pronounced in the latter. The present data do not establish whether the hemostatic effect of EACA was by suppression of urokinase or blood lytic activity. If the latter mechanism is more important, EACA may prove of value as a hemostatic following other types of surgery.

Localization of the Renal Tubular Effect of Parathyroid Hormone. A. H. Samiy, P. F. Hirsch and A. G. Ramsay, Boston, Mass. (introduced by M. W. Ropes).

A series of experiments was performed to study the effect of parathyroid hormone on the excretion of phosphate in parathyroidectomized dogs, utilizing the stop flow technique of Malvin and co-workers, before and after a single intravenous injection of 200 to 400 U of a purified parathyroid extract. Injection of the extract produced a significant phosphaturia without increasing the filtered load of phosphate. The increase in phosphate excretion resulted from a decrease in proximal reabsorption. There was no evidence of a net tubular secretion of phosphate during the free flow clearances and the stop flow periods. The excretion of phosphate did not exceed the filtered load of phosphate in any of the experiments. Furthermore, $U/P_{PO_4} : U/P_{Creat.}$ ratios were less than 1.0 in all of the stop flow experiments. Further experiments were performed to study the effect of the parathyroid extract on the transtubular transport of phosphate. P^{32} was injected with inulin 1 minute before releasing the ureteral clamp during a stop flow experiment. A small net flux of P^{32} into the tubular fluid was demonstrable. The injection of parathyroid extract did not increase significantly the net transtubular flux of P^{32} despite an accompanying increase in phosphate excretion. These studies indicate that the phosphaturic action of the parathyroid extract results from an inhibition of tubular reabsorption of phosphate and not from any significant effect on the secretion of phosphate.

The Effects of "Dry" Heat on the Circulation of Man. V. Coronary Hemodynamics. Salvatore M. Sancetta, Elmerice Traks, Donald B. Hackel and Benjamin Wittels, Cleveland, Ohio (introduced by Charles H. Rammelkamp, Jr.).

The deleterious influence of hot weather on cardiac patients is well known, but data relevant to the changes resulting from a short-term "dry" heat exposure in resting man are lacking. The effects of a 2 hour exposure to a relatively dry, hot atmosphere of 40 per cent humidity and 98° F on left ventricular myocardial blood flow (coronary sinus catheterization, nitrous oxide saturation-desaturation) were studied in 24 hospitalized, resting patients, equally divided into three groups: subjects with normal hearts, with enlarged left ventricles but not in failure (normal pulmonary arterial wedge pressure on cardiac catheterization), and with enlarged left ventricles in failure (elevated pulmonary arterial wedge pressure). The baseline determinations were obtained at the comfortable environment of 40 per cent humidity and 73° F. An additional 8 control subjects were studied in the comfortable environment only. The subjects in all three groups showed significant increases in the heart rate and decreases in the intrabrachial arterial pressure. Coronary blood flow and arteriovenous differences decrease variably, though not significantly, when compared with the control patients in whom no change occurred. Coronary vascular resistance and per cent myocardial oxygen extraction did not change. The decreased coronary blood flow was caused by the reduced perfusion pressure and, more importantly, by the decreased work demand of the heart, as evidenced by a significant reduction in left ventricular myocardial oxygen consumption in all three groups. The decrease noted in all groups was also significant when compared with an over-all increase that occurred in the control subjects. This demonstrates that in the absence of restlessness and motor activity, the normal, as well as the diseased, left ventricle may tolerate a short-term exposure to hot dry weather, aside from those situations where a decreased perfusion pressure may be expected to be injurious because of purely physical and hydrostatic considerations.

New Pathway of Steroid Metabolism in Man. Avery A. Sandberg,* W. Roy Slaunwhite, Jr. and M. Neeman, Buffalo, N. Y.

Existing concepts of the metabolism of steroidal 4-ene-3-ones in human subjects indicated that the major pathways involve the following sequential steps: reduction of the unsaturation at positions 4,5 in ring A;

reduction of the 3-keto group; and conjugation of the resulting hydroxyl group, mainly with glucuronic acid. Experiments performed in our laboratory demonstrate a new pathway of steroid metabolism for adrenal steroids: 11β-hydroxyandrostenedione (I) and its 11-keto analog adrenosterone (II) were administered orally or intravenously as tracers labeled with C^{14}, with and without large amounts (1 to 3 g) of carrier steroid. After administration of I or II, the steroid conjugates excreted in the urine were hydrolyzed by enzyme-mild acid, affording a metabolite of low polarity in about 5 per cent yield, which has been proven to be 3,5-androstadiene-11β-ol-17-one (III). Its 11-keto analog was found after administration of II. Since the new metabolite appeared in the urine as a conjugate, its precursor probably was a 4-ene-3α-ol, easily dehydrated during the enzymatic hydrolysis at pH 5. Indeed, hydrolysis of urine at neutral pH with bacterial β-glucuronidase did not afford the 3,5-diene, which was produced by warming the steroid extract with acetic anhydride. Oxidation of an aliquot of the extract with hydrated manganese dioxide produced I. These findings prove unequivocally that the precursor of III is a 4-ene-3α-ol, probably conjugated with glucuronic acid. It thus appears that some steroidal 4-ene-3-ones can first undergo reduction of the 3-ketone group, followed by conjugation, leaving the Δ^4-unsaturation intact. It is possible that this pathway plays an important role in the metabolism of other steroids, or in certain abnormal conditions, though its occurrence may not have been detected due to the instability of allylic 4-ene-3-ols in conventional hydrolytic procedures.

The Hematotoxicity of Amphotericin B. J. P. SANFORD, J. A. PRITCHARD, R. E. WINDOM, R. S. ABERNATHY AND H. G. MUCHMORE, Dallas, Tex., Little Rock, Ark. and Oklahoma City, Okla. (introduced by Gladys J. Fashena).

Amphotericin B is the most effective agent against systemic fungal infections. While its usefulness has been limited by febrile reactions and by nephrotoxicity, hematotoxicity seldom has been recorded and generally is stated not to occur. However, decreases in hemoglobin concentrations of $\geqslant 2.0$ g per cent have been observed in 24 of our 32 patients receiving amphotericin B. This anemia, which is associated with hypoferremia, has not been accompanied by leukopenia or thrombocytopenia. In patients receiving amphotericin B, the anemia is related to therapy and not to the underlying fungal infection, since it develops despite clinical improvement, occurs in individuals with superficial fungus infections who receive amphotericin B, and remits when the drug is stopped. Likewise, the anemia does not seem to be the consequence of azotemia, since it precedes and even may occur without azotemia. These studies were undertaken to ascertain the mechanism of the anemia. Hemodilution, external bleeding, or development of incomplete or drug-dependent circulating antibodies were excluded by appropriate studies. Moderate increases in RBC destruction as measured by Cr^{51}-RBC survival were observed in only 2 of 10 patients; likewise, increases in fecal or urine urobilinogen or in serum bilirubin were not observed. Ferrokinetic studies demonstrated normal or slightly accelerated disappearance of Fe^{59} from plasma and a normal rate of incorporation of transferrin-bound Fe^{59} into RBC. These ferrokinetic data exclude suppression of RBC production of the type noted with chloramphenicol but are compatible with a defect in the transfer of iron from nonviable RBC to the plasma transferrin pool. This block, coupled with unimpaired removal of iron from plasma transferrin, results in hypoferremia. A similar mechanism has been proposed for the anemia associated with turpentine-induced inflammation. Thus, the anemia associated with amphotericin B is postulated to be the consequence of defective reutilization of iron.

Kinetics of Intestinal Absorption in Man: Normal Subjects and Patients with Sprue. HAROLD P. SCHEDL AND JAMES A. CLIFTON, Iowa City, Iowa (introduced by William B. Bean).

Absorption kinetics were studied at various levels by transintestinal intubation. Solutions pumped into the oral end of the tube entered the intestine at a known site; 15 cm distally the perfusate was aspirated and collected from the anal end of the tube. Polyethylene glycol was used as a nonabsorbable indicator. In 4 normal subjects absorption rates of glucose, D(+)-xylose, and cortisol decreased from jejunum to ileum. Absorptions of xylose (jejunal 17 ± 7 and ileal 4 ± 2 per cent per hour per 15 cm gut) and cortisol (jejunal 53 ± 10 and ileal 35 ± 14 per cent per hour per 15 cm gut) were proportional to concentration. The glucose absorption-concentration relation was a rectangular hyperbola. Absorption approached a maximum: jejunum, $V_{max} = 5.6$ g per hour per 15 cm, the Michaelis constant, K_M, was 20 mmoles; ileum, $V_{max} = 3.3$ g per hour per 15 cm, $K_M = 23$ mmoles. In a patient with active sprue the absorption gradient was absent: glucose absorption rate (1 per cent solution) was 1 g per hour per 15 cm, $V_{max} = 1.7$ g per hour per 15 cm, $K_M = 29$ mmoles, in both jejunum and ileum. Cortisol (100 mg per study) restored the absorption gradient and raised the jejunal and ileal V_{max} to 5.0 and 3.7 g per hour per 15 cm, respectively. Absorption rates for 1 per cent solutions were normal: jejunal 4 and ileal 2.5 g per hour per 15 cm. K_M remained 25 to 30 mmoles. On cortisol treatment jejunal cortisol absorption rose from 21 per cent (cortisol-4-C^{14}) to 54 per cent, but ileal absorption was unchanged, 33 vs 35 per cent. Likewise, jejunal xylose absorption rose from 1 to 7 per cent but ileal absorption was unchanged, 3 and 4 per cent vs 2 per cent. After 3 months on a gluten-free diet without steroids, absorption was normal. Another patient with active sprue had jejunal absorption of 0.7 g per hour per 15 cm for 1 per cent glucose, $V_{max} = 1.8$ g per hour per 15 cm, $K_M = 35$ mmoles. A third sprue patient in spontaneous remission had normal glucose absorption.

Steroids did not affect absorption. The K_M, 90 mmoles, differed from that of all other persons studied, including a patient with Whipple's disease.

Detection of Genetic Carriers of Spherocytosis. R. F. SCHILLING,* A. A. MACKINNEY, N. STERNSCHUSS AND N. E. MORTON, Madison, Wis.

Published data concerning this presumably simple dominant hereditary defect indicate a shortage of affected individuals. Possible explanations for this include failure to diagnose mild cases, mutation, and early death of affected persons. To test the possibility that mildly affected persons might better be detected by multiple laboratory tests, we examined 50 normals and 175 members of 26 spherocytotic families. The following were determined: reticulocytes, hemoglobin, blood smear for spherocytes, bilirubin, mechanical fragility, osmotic fragility, and autochemolysis. Using an electronic computer, a discriminant was formed to maximize the intraclass correlations between the normals and 29 patients having classical hereditary spherocytosis. This discriminant was used to classify family members as normal or affected. A physician reviewed the data and diagnosed family members independently. Of 106 members studied with complete discriminant there were three disparities between the "computer classification" and that of the physician. When using a simple discriminant based on hemoglobin, reticulocytes, blood smear, and bilirubin to study 168 family members there were five disparities. Using multiple laboratory tests improves diagnostic accuracy over that of a single test and the family history. The history is a poor discriminant for classifying family members: incidence estimated by history was only two-thirds as large as by laboratory. In completely examined sibships there is no shortage of affected individuals from the expected 0.5 for a dominant trait with high penetrance. In this material there are four isolated probands having normal parents. These may represent mutants or isolated cases of unrelated hemolytic anemia.

The Kinetics of Simultaneously Administered C^{14}-bicarbonate, Na^{24} and T-1824 in Man. ROBERT SCHWARTZ, ROGER C. DEMEUTTER AND WALTON W. SHREEVE, Cleveland, Ohio and Upton, N. Y. (introduced by Frederick C. Robbins).

The metabolism of labeled bicarbonate was studied in order to obtain data on the movement and distribution of $C^{14}O_2$ within the body fluids following the metabolism of a C^{14}-labeled compound. A single injection, containing $HC^{14}O^-_3$, Na^{24} and T-1824, followed by a continuous infusion of the $HC^{14}O^-_3$ for 30 to 60 minutes, was given to each subject (adult diabetics). Arterial and venous blood samples were taken simultaneously from the forearm and breath samples were collected for $C^{14}O_2$ analysis. Circulatory mixing and plasma volumes were determined from the dye dilution curves of T-1824 in plasma. Na^{24} similarly served as an indicator of mixing in the interstitial fluid during the first hour. The time course of the specific activity (SA) of the arterial $C^{14}O_2$ was similar to that of Na^{24} and both described smooth curves which maintained plateaus between 20 and 60 minutes. Breath and arterial $C^{14}O_2$ SA's were identical. Venous Na^{24} and $C^{14}O_2$ SA's increased variably and gradually approached or reached a course parallel to the arterial curves within 20 to 60 minutes. Arteriovenous differences in SA were greater for $C^{14}O_2$ than for Na^{24} during the infusion and cannot be entirely accounted for by metabolic (nonlabeled) CO_2 production. The apparent bicarbonate pool, calculated by integration of the arterial-breath SA curves at intervals during infusion, increased significantly. About one-third of the total administered C^{14} was expired during the infusion period and one-third the following 6 hours. Turnover time ($1.44 \times T_{\frac{1}{2}}$) of bicarbonate calculated from the SA of $C^{14}O_2$ in the breath was about 60 minutes during the first hour after infusion. Thereafter, it increased progressively. These data suggest exchange of circulating bicarbonate with a large pool of organic or inorganic constituents (e.g., possibly urea or bone carbonate) which has a relatively slow turnover.

Evidence for a Renal Tubular Amino Acid Transport System Common to Glycine, L-Proline and Hydroxy-L-proline. CHARLES R. SCRIVER, IRWIN A. SCHAFER AND MARY L. EFRON, Montreal, Canada and Boston, Mass. (introduced by John C. Beck).

An operational definition of amino acid transcellular transport at present must consider factors including Schiff-base formation with pyridoxal-5-phosphate, potassium exchange, energy supply and genetic control. A transport mechanism may be selective for some, and exclusive for other, amino acids; cystinuria is the preeminent example demonstrating a selective, genetically controlled, transport system. There is now evidence for another selective amino acid transport system; it is common to glycine, L-proline and hydroxy-L-proline. The following data support this hypothesis. 1) Intravenous amino acid infusions in 7 normal subjects: Equimolar amounts of L-proline (7 subjects), glycine (3) and hydroxy-L-proline (1) were infused intravenously (3-minute period) on separate occasions. Fasting amino acid clearances were calculated in consecutive control (30 minute) and 3 postinfusion (15, 30 and 30 minute) periods. Quantitative amino acid data were obtained using cation exchange resin elution column chromatography; qualitative confirmation of results was obtained by two-dimensional paper partition chromatography. Infusion of either imino acid in the triad produced significant increments in the renal clearance of the other two amino acids, persisting while the filtered load of the infused amino acid was increased. Glycine produced proline excretion in only one subject. The remainder of the filtered amino acids was unaffected. The transport system exhibited higher affinity for the two imino acids, thus correlating with known fasting clearance rates for glycine (high), proline (low) and hydroxypro-

line (low). 2) Studies in disorders of amino acid metabolism: Urine and plasma amino acid data in *a*) adolescent osteomalacia and renal glycinuria, *b*) Hartnup disease, and *c*) familial hyperprolinemia (newly discovered by the authors) gave supporting evidence for the renal tubular amino acid transport system under discussion. There is therefore strong evidence for a renal tubular transport mechanism common to glycine, L-proline and hydroxy-L-proline; structural and biological properties of these amino acids stimulate interesting speculations about the significance of this observation.

Regulation of Galactose Metabolism in Hemolysates from Normal, Heterozygous and Galactosemic Subjects. STANTON SEGAL, T. DAVID ELDER AND YALE J. TOPPER, Bethesda, Md. (introduced by Wallace P. Rowe).

Progesterone and menthol stimulate the metabolism of a tracer dose of galactose-1-C^{14} in galactosemic subjects by an unknown mechanism. The rate of epimerization of UDP-galactose can regulate the conversion of galactose to CO_2 by normal tissues *in vitro*. The present experiments were undertaken to ascertain whether stimulation of the rate of epimerization and subsequent reactions can accelerate galactose oxidation in systems wherein P-gal-transferase is diminished. Oxidation of galactose-1-C^{14} to $C^{14}O_2$ in hemolysates fortified with excess ATP and TPN has been studied. Stimulation of endogenous epimerase by removal of DPNH, addition of purified epimerase or of phosphoglucomutase to the normal increased galactose oxidation substantially. The same per cent increase in oxidation was observed in hemolysates having a diminished capacity to oxidize galactose due to a reduced P-gal-transferase level (blood from heterozygous subjects or mixtures of normal and galactosemic bloods). This has been demonstrated even in mixtures where the capacity to oxidize galactose was less than 10 per cent of that of the normal hemolysate, and also when the systems were saturated with galactose. No galactose was oxidized to CO_2 by galactosemic hemolysates under any of these conditions. These results demonstrate that transferase present in hemolysates does not function at maximal capacity even when its level imposes a profound limitation on the rate of galactose oxidation. The turnover number of this enzyme can be increased by accelerating removal of the two products of this reaction, UDP-galactose and glucose-1-phosphate. If small amounts of transferase are present in other tissues of galactosemic subjects it is conceivable that this deficient enzyme activity may be increased as a result of stimulation of reactions subsequent to the metabolic block.

A Copper-protein of Human Liver. JAY SHAPIRO, ANATOL G. MORELL AND I. HERBERT SCHEINBERG,* New York, N. Y.

The livers of patients with Wilson's disease contain much more copper than those of control subjects. Although several investigators believe that the hepatic pathology which characterizes this disease is caused by excessive copper, little is known of the nature either of this copper or of the small amount that is normally present. Our investigation of this problem has led to the purification of a copper-protein from the livers of control subjects and of patients with Wilson's disease. Following homogenization of fresh or frozen liver, and ultracentrifugation and dialysis of the supernatant, the solution of the copper-protein is purified by column chromatography using diethylaminoethyl cellulose and hydroxyapatite as adsorbents. The yield of the protein is 2 mg per g of wet normal liver and about 2 to 8 mg per g of wet liver from patients with Wilson's disease. The protein 1) has a molecular weight of about 10,000; 2) is electrophoretically homogeneous at pH 8.6; 3) has essentially the same amino acid analysis whether isolated from patients or control subjects; 4) has a unique ultraviolet absorption spectrum resulting from its copper content; and, 5) exhibits no oxidase activity against paraphenylenediamine, ascorbic acid, dihydroxyphenylalanine or cytochrome C. Copper constitutes, by weight, 0.1 to 0.3 per cent of the protein from livers of control subjects, and up to 3 per cent of the protein from livers of patients with Wilson's disease. This variation in copper content appears to be the only definite difference between the proteins isolated from control subjects or these patients. Finally, the copper in the protein is in the uncommon cuprous form, and is reversibly bound to sulfhydryl groups.

Prevalence of Human Infection with Vesicular Stomatitis Virus. ALEXIS I. SHELOKOV, PAULINE H. PERALTA AND PEDRO GALINDO, Balboa Heights, Canal Zone and Panama City, Republic of Panama (introduced by Joseph E. Smadel).

Vesicular stomatitis (VS), an important disease of farm animals in the western hemisphere, is caused by two distinct virus types: Indiana and New Jersey. Antibodies can be demonstrated in exposed farmers and laboratory personnel. Documented laboratory infections have been associated with influenza-like symptoms. Because of seasonal occurrence of the animal disease, its natural transmission by an arthropod vector has been postulated. During the first 12 months of a 3-year project on the ecology of arthropod-borne viruses in a tropical rain forest, which is being conducted by the Gorgas Memorial Laboratory with the collaboration of the Middle America Research Unit, we isolated two strains of Indiana VS virus from phlebotomus sandflies. Examination of 490 sera from long-time residents of a nearby community disclosed neutralizing antibodies in 27 per cent. Percentage of positive reactors increased from 10 per cent under age 10 to 35 per cent at age 30 and older. Of the 130 neutralizing sera, 28 (22 per cent) were reactive with Indiana VS virus antigen by complement fixation. On sampling of several other Panamanian communities, differences were found in the local prevalence of neutralizing antibodies, ranging from none in the urban to 35 per cent in the forested areas. Prevalence of human antibodies and isolation of the virus from biting arthropods form the basis for a field and labora-

tory investigation of this infection as a public health problem.

Kinetics of Reactions Between a Prothrombin Derivative and Proteolytic Enzyme Inhibitors. N. RAPHAEL SHULMAN * AND JOHN Z. HEARON, Bethesda, Md.

Soybean trypsin inhibitor and an inhibitor separated from plasma are known to act as anticoagulants, but the mechanism and site of their action have been controversial. Kinetic analysis of the effects of varying the concentrations of inhibitor, purified prothrombin and biological activators (thromboplastin, accelerator, Ca^{++}) on the final yield of thrombin indicated that the inhibitors do not react with thrombin or with any substance present in the system before prothrombin conversion occurs. Rather, a complex is formed between inhibitor (I) and a derivative (D) in the pathway of thrombin (T) formation. The complex cannot be reversed by biological activators of prothrombin (P), but I in the D-I complex (C) can be neutralized with an equimolar amount of trypsin which, by virtue of its greater affinity for I, displaces D. The trypsin-I complex thus formed is inert, and the subsequent conversion of regenerated D to T is dependent on biological activators. The following model and equation describe the properties of this unusual form of inhibition in which an intermediate derivative of a proenzyme participates in two essentially irreversible, competitive-rate reactions:

$$P \rightleftharpoons D \begin{cases} \xrightarrow{k} T \\ \xrightarrow[rI]{} C \end{cases}; \qquad C_\infty = I_0 P_0 / [I_0 + (k/r)]$$

where C_∞ is a final concentration and I_0 and P_0 are initial concentrations of reactants, k is a first order rate constant dependent on concentration of activators, and r is a second order rate constant for combination of I and D. The system may operate to control blood coagulation, for low activator activity (low k) which may occur intravascularly would favor C formation and prevent T formation, whereas high activator activity (high k) which occurs extravascularly would favor T formation. Similar kinetics may be applicable in other systems involving activation of enzymes in the presence of inhibitors (e.g., fibrinolytic system).

Treatment of Nontoxic Nodular Goiter with Triiodothyronine: A Double Blind Study. Joseph E. Sokal, KATSUTARO SHIMAOKA, JORGE MADILLO AND FRANK C. MARCHETT, Buffalo, N. Y. (introduced by David K. Miller).

Despite extensive discussion over many years, the management of nontoxic nodular goiter remains a highly controversial subject. Among the points in major dispute is the response of such goiters to thyroid medication. One group has reported that decrease or disappearance of goiter occurred in every one of 36 patients treated with triiodothyronine. Others have stated that treatment with thyroid preparations so rarely results in decrease of goiter that it is virtually useless. In order to obtain unbiased data on this point, we are conducting a double blind study of the effect of 75 μg of triiodothyronine daily on nontoxic nodular goiters. Patients receive active drug or placebo by random allocation and are examined periodically by an internist and a surgeon. Each examiner records his observations privately and neither knows the results of periodic determinations of I^{131} uptake and serum PBI. Whenever possible, each patient serves as his own control, receiving placebo for 3 months and triiodothyronine for 3 months, with a 1-month rest period between courses. To date, 26 patients have completed both triiodothyronine and placebo courses, 10 patients have completed triiodothyronine courses only, and 5 patients have completed placebo courses only. Depression of I^{131} uptake or PBI was seen in three-fourths of the triiodothyronine courses. Side effects such as nervousness, palpitations, tremor, and heat intolerance were seen during both placebo and triiodothyronine treatment. The two examiners usually agreed on the effect of treatment on goiter size. Decrease in goiter occurred during 8 per cent of the placebo courses and 42 per cent of the triiodothyronine courses. Two goiters increased in size during triiodothyronine treatment; one of them was malignant.

The Diagnostic and Prognostic Category, Suspect Coronary Heart Disease. JEREMIAH STAMLER,* HOWARD A. LINDBERG AND DAVID M. BERKSON, Chicago, Ill.

A key problem confronting clinical investigation today is the diagnosis of disease in its early, incipient or suspect stages. This is particularly important when incidence and mortality from frank disease are high and when recent research advances offer the possibility of primary and secondary prevention. These conditions prevail conspicuously today with respect to atherosclerotic coronary heart disease (CHD). The present study, begun on January 1, 1958, involves the long-term follow-up of 1,594 men aged 40 to 59, the entire male labor force of this age employed by a Chicago utility company. Data collected at 1 to 2 year intervals demonstrate high prevalence rates for such coronary risk factors as hypercholesterolemia, hypertension, obesity, heavy smoking, positive family history (singly or in combination). Definite CHD was found in 48 per 1,000 men. The category, suspect CHD, was used to designate men with suspect (but not definite) angina pectoris, with suspect (but not proved) episodes of myocardial infarction or coronary insufficiency, with certain electrocardiographic abnormalities not pathognomonic of definite CHD. Men with suspect CHD have a 2 to 3-fold greater risk of developing frank CHD in middle age than men free of any clinical signs of CHD. The over-all prevalence rate of suspect CHD was high (111 per 1,000), principally due to suspect angina pectoris or ECG findings. The prevalence rate of suspect CHD was significantly higher in hypercholesterolemic than normocholesterolemic, hypertensive than normotensive, obese than nonobese men. In men with combinations of these abnor-

malities, suspect CHD prevalence rate was in the range 151 to 333 per 1,000, with 4 to 8-fold increase in risk compared with normal men. These findings lend support to the concept that suspect CHD is a valid diagnostic and prognostic category. It would seem advisable in men with suspect CHD to institute prompt measures in an attempt to correct risk-increasing abnormalities and to achieve possible prevention of frank CHD.

Insulin Content of Pancreas from Nondiabetic and Diabetic Subjects and from Pancreatic Islet Cell Tumors, as Extracted on Cationic Exchange Resin and with Acid Ethanol. JURGEN STEINKE, ALBERT E. RENOLD,* JURGEN KNAACK AND HARRY N. ANTONIADES, Boston, Mass.

We have previously reported that a portion of the insulin contained in homogenates of pancreas can be adsorbed on cationic exchange resin at physiologic pH, while crystalline insulin remains unadsorbed. This suggests association of insulin in pancreas with cationic protein(s), probably representing the storage form(s) of insulin. Anomaly of this association is one of several possible pancreatic defects in diabetes. Accordingly, pancreatic insulin content has been measured subsequent to *a*) acid ethanol extraction (Scott and Fisher); and *b*) the cationic resin extraction procedure. Insulin was assayed by the $C^{14}O_2$ rat epididymal adipose tissue technique. Acid ethanol extraction of pancreas from 12 nondiabetic patients (mean age, 61 years), 6 growth-onset diabetics (onset between 7 and 17 years), and 7 patients with maturity-onset diabetes (mean age, 73 years) revealed insulin contents of 1.78 ± 0.25; less than 0.1; and 1.12 ± 0.16 U per g. In the same tissue, resin-adsorbable insulin content was 1.82 ± 0.29; less than 0.07; and 1.04 ± 0.21 U per g, respectively. In addition, the pancreatic homogenates contained 14, 13, and 16 per cent resin nonadsorbable insulin. These values agree well with those obtained by Scott and Fisher, and by Wrenshall, using a mouse convulsion assay procedure. The data suggest that in diabetic, as in normal pancreas, most if not all insulin is present in a complex form, associated with basic protein(s). Five islet cell tumors were extracted with resin and shown to contain between 2.1 and 24.1 U insulin per g, with a nonadsorbable component averaging 16 per cent. In one patient the "normal" pancreas adjacent to the tumor contained 0.09 U per g of tissue, a value in the juvenile diabetes range. This patient exhibited severe and prolonged postoperative hyperglycemia.

Estimation of Free Thyroxine Concentration in Serum. KENNETH STERLING,* New York, N. Y.

The demonstration of "free" or "unbound" thyroxine in serum by dialysis through cellophane has recently been reported, with identification of the hormone on chromatographic analysis of the dialysate (Sterling and Tabachnick). Further efforts to measure this minute moiety have led to the development of a technique employing the cation exchange resin Dowex 50W-X8 for the separation of thyroxine and iodide in the dialysate (Sterling, Tabachnick and Hegedus). The validity of the procedure was confirmed by chromatography. In the present work the sera of thyrotoxic subjects were studied by two methods: 1) dialysis of endogenously labeled sera 1 to 7 days after I^{131} therapy, and 2) addition of I^{131}-labeled thyroxine to sera in tracer amounts, such that the increment in thyroxine iodine concentration was 0.2 μg per 100 ml or less, a physiologically insignificant change. The two methods gave similar results; consequently, various other sera were investigated by the second method. The results obtained for free thyroxine concentration were of the order of 10^{-10} M. These values were in reasonable agreement with previous computations by Robbins and Rall and with recent data from this laboratory indicating four thyroxine binding sites on the albumin molecule (lysyl epsilon-amino groups) with an apparent association constant of approximately 10^5. In the various categories studied, the free thyroxine values, expressed as per cent of the total thyroxine content of the sera, were as follows (mean $\pm$ SD): thyrotoxicosis, 0.37 ± 0.09 per cent; euthyroidism, 0.26 ± 0.02 per cent; myxedema, 0.17 ± 0.03 per cent, pregnancy or estrogen administration, 0.16 ± 0.07 per cent. The findings are compatible with the assumption that physiological activity depends upon the unbound hormone.

Metabolic Changes in Normal and Glucose-6-Phosphate Dehydrogenase-Deficient Erythrocytes Induced by Acethylphenylhydrazine. NECHAMA STERNSCHUSS, GRACE A. VANDERHOFF, ERNST R. JAFFÉ AND IRVING M. LONDON,* New York, N. Y.

These studies are concerned with the mechanism by which glucose-6-phosphate dehydrogenase (G6PD) deficiency may result in accelerated destruction of erythrocytes. Washed erythrocytes of normal individuals and of Negro males with G6PD deficiency were incubated (37° C) in phosphate buffer with glucose, 200 mg per 100 ml, and with or without acetylphenylhydrazine (APH), 250 mg per 100 ml. In the presence of APH, the reduced glutathione (GSH) of normal erythrocytes declined from 67 (51–86) to 36 (23–51) mg per 100 ml after 7.5 hours, while the GSH of G6PD-deficient erythrocytes decreased from 46 (33–64) to 3 (0–6) mg per 100 ml after 2.5 to 5 hours. In the presence of APH, a significant difference in glycolysis was demonstrable between normal and G6PD-deficient cells: lactate production was diminished by only 10 per cent in normal cells as compared to 30 to 50 per cent in G6PD-deficient cells after 7.5 hours' incubation. Pyruvate accumulation did not occur in the absence of APH, but occurred in both types of cells in the presence of APH. The accumulation of pyruvate could account for the diminished lactate production in normal but not in G6PD-deficient cells. No diminution in the potential activity of glyceraldehyde-3-phosphate dehydrogenase or of lactic dehydrogenase was

demonstrable by assay of these enzymes. Furthermore, on addition of reduced diphosphopyridine nucleotide (DPNH) to lysates of both types of cells, pyruvate was rapidly converted to lactate. Although methemoglobin and sulfhemoglobin accumulated in the presence of APH, the two types of erythrocytes did not differ in the extent to which their hemoglobin was altered. These findings indicate that 1) APH treatment results not only in a diminution of GSH but also in a deficiency in the amount or availability of DPNH, and 2) that glycolysis as measured by lactate and pyruvate production is diminished in G6PD-deficient cells treated with APH.

Regenerative Macrocytosis in Experimental Phenylhydrazine Anemia. FREDERICK STOHLMAN, JR.* AND GEORGE BRECHER, Bethesda, Md.

The production of severe anemia in the rat by phenylhydrazine is followed by an increase in reticulocytes within 48 hours, reaching a maximum in 4 to 5 days. Five days after phenylhydrazine, reticulocyte values of 90 to 100 per cent are present. These cells are abnormal; the mean MCV is 100 to 110 (normal 55), MCHC 27 to 31; size distribution curves indicate that a significant number of cells have an MCV of as much as 150. The survival of these reticulocytes, measured in irradiated and hypertransfused animals, may be as long as 5 days compared with the normal of 2 to 3 days; the survival of the red cells derived from these reticulocytes, however, is shortened, 40 per cent of them being destroyed by Day 20. Following the peak reticulocytosis, size distribution curves suggest the elimination of the larger cells as a possible explanation for the shortening of the total red cell life span. Continued elevation of MCV (80 to 90) with return of the MCHC to normal after Day 8 argues against shrinkage as the cause for the loss of these larger cells. The early release of large numbers of reticulocytes in response to severe anemia might be explained by a shortened generation time or, more likely, skipped divisions within the erythroid compartment. The continued production of reticulocytes in rats with phenylhydrazine anemia, when given lethal doses of radiation (650 r, which produces complete aplasia in the normal rat), favors but does not prove the differentiation of substantial numbers of stem cells into erythroid elements which mature without dividing.

The Effect of Changes in Diet on Fat Mobilization and Transport in Man. JAMES M. STORMONT AND CHRISTINE WATERHOUSE,* Rochester, N. Y.

Variations in fasting plasma lipid components were induced by changes of diet composition in 9 subjects (3 control, 3 obese, 2 terminal carcinoma, 1 subacute cirrhosis). Each subject was placed on a metabolic ward and three diets were given: *a*) low calorie (700 calories); *b*) high carbohydrate (75 per cent carbohydrate, 1,700 calories); and *c*) high fat (75 per cent fat isocaloric with *b*). After 1 week on a constant diet, subjects were fasted 15 hours and plasma was obtained for measurement of free fatty acid (FFA), triglycerides, cholesterol and cholesterol esters. While fasting FFA concentrations were elevated following low calorie diet (range 0.81 to 2.29 mEq per L), fasting FFA levels fell in all subjects after 1 week of high carbohydrate diet (range 0.30 to 0.94), and rose when fat was substituted isocalorically for dietary carbohydrate (range 0.59 to 1.87 mEq per L). FFA concentrations in patients with cirrhosis and obesity were higher than others. Fasting plasma triglycerides rose in control and obese patients following high carbohydrate diet and fell following high fat diets. In patients with carcinoma and cirrhosis, triglycerides fell following high carbohydrate diets. Cholesterol esters rose slightly in obese patients after high carbohydrate diets but fell in other subjects. A rise in cholesterol esters occurred in all subjects following high fat diets. Oral administration of trace amounts of palmitic acid-1-C^{14} after each diet in a patient with carcinoma revealed no evidence of delayed incorporation of label into triglycerides; however, incorporation into cholesterol and cholesterol esters was delayed following high fat diets. The time course of specific activity for triglyceride and FFA was similar under all dietary conditions. These results further delineate dietary-induced adaptations in fat metabolism, and suggest specific changes in fat mobilization, transport and synthesis with alteration in diet, and with certain disease states.

Autoregulation of Intestinal Blood Flow. E. CLINTON TEXTER, JR., STEVEN MERRILL, MELVIN SCHWARTZ, GUIDO VAN DERSTAPPEN AND FRANCIS J. HADDY,* Chicago, Ill.

It has long been thought that the kidney was unique in its ability to hold its blood flow constant despite fluctuations in arterial pressure over the approximate range 70 to 200 mm Hg. However, Johnson recently reported that intestinal blood flow did not fall as much as expected when arterial pressure was lowered from normal to 40 mm Hg. Since this suggested that the intestinal circulation might also have an efficient local mechanism for regulating its flow, we have examined the relationship of pressure to flow in the intestinal bed of the dog over more extensive ranges and have compared this relationship with those obtained by the same method in the kidney and forelimb. A pump was interposed in the superior mesenteric artery, and pressures were measured in the superior mesenteric artery, a small artery (0.5 mm), a small vein (0.5 mm) and the portal vein as flow was increased over the range 20 to 360 ml per minute. In each of 10 experiments, pressures in the superior mesenteric and small arteries increased more rapidly than flow over the approximate range 90 to 270 ml per minute. Total resistance rose from 0.57 mm Hg per ml per minute at a pressure of 64 mm Hg to 0.76 at a pressure of 205 mm Hg. Corresponding values for resistance to flow through small vessels (less than 0.5 mm) were 0.26 at 39 and 0.53 at 150. These changes are similar to those obtained

in the kidney but differ from those obtained in the forelimb. These findings indicate that autoregulation is equally efficient in the intestinal and renal beds and that the regulation is accomplished by active vasomotion in vessels less than 0.5 mm diameter.

The Distribution of Iron Injected Intraperitoneally: Evidence of Serosal Absorption by the Small Intestine. PHAIROJANA THIRAYOTHIN, Washington, D.C. (introduced by William H. Crosby).

Three days after the intraperitoneal injection of 50 mg of iron as iron-dextran, much iron had accumulated as ferritin in the liver and spleen. (The animals were 200 g albino rats of the WRCF strain. The method of estimating ferritin was a micro-modification of Granick's $CdSO_4$ technique for crystallization of ferritin from tissue homogenates.) Ferritin could also be recovered from the intestine, especially the duodenum and upper jejunum. There was none in the stomach and colon. Ten days after injection ferritin could not be recovered from the gut, though it was present in heavy concentration in liver and spleen. When the iron was injected intravenously or subcutaneously in heavier doses (150 mg) it went to the liver and spleen without preliminary accumulation in the small intestine. In the significant experiment a loop of small intestine at the duodenojejunal junction was brought out of the peritoneum and tacked under the skin. Two weeks were allowed for healing of the wound. Iron-dextran was then injected intraperitoneally and after 3 days the animals were sacrificed. Ferritin was found in the small intestine adjacent to the extraperitoneal loop, but no ferritin was found in that loop. It is concluded that the ferritin which is present in the small intestine following intraperitoneal injection of iron-dextran is derived from iron or iron-dextran which migrates through the serosal surface of the intestine.

The Influence of Arterial Pressure on the Antihypertensive Action of a Normal Kidney, a Biological Servomechanism. LOUIS TOBIAN,* BEVERLY WINN AND JEANETTE JANECEK, Minneapolis, Minn.

An isolated normal kidney was connected to the carotid artery and jugular vein of rats with "Goldblatt" hypertension and was perfused for 3 hours. In one group of 22 hypertensive rats. the arterial connection was made with a short length of polyethylene tubing so that the isolated normal kidney was perfused with elevated pressures (around 203 mm Hg). In this group the blood pressure of the hypertensive rats fell 67 per cent of the way toward normal in 1.75 hours and 90 per cent of the way toward normal in 2.5 hours. In another group of 20 equally hypertensive rats, the arterial connection was made with a long length of tubing so that the isolated normal kidney was perfused with normal pressures (around 90 mm Hg). In this group the blood pressure of the hypertensive rats fell only 15 per cent of the way toward normal in 1.75 hours and only 35 per cent of the way toward normal in 2.5 hours. These differences were highly significant ($p < 0.00005$). In 8 other hypertensive rats, "nephrosclerotic" kidneys were used as the isolated kidney and were perfused with elevated pressures. They were able to bring the blood pressure down toward normal almost as effectively as normal kidneys. Six sham perfusions using a polyethylene arteriovenous fistula without any isolated kidney in the circuit induced only a 10 per cent reduction of arterial pressure toward normal in 1.75 hours and only a 14 per cent reduction in 2.5 hours. In summary, the antihypertensive mechanism of a normal kidney is brought into action when the kidney is perfused with elevated blood pressures. When it is perfused at normal pressures, the antihypertensive action receives little or no stimulation. This appears to be a feed-back arrangement designed to maintain normal arterial pressures.

Patterns of Estriol Conjugates in Body Fluids of the Human Fetus and Newborn. PHILIP TROEN AND EGON DICZFALUSY, Stockholm, Sweden (introduced by Herrman L. Blumgart).

It has been demonstrated previously that the estrogen pattern of cord blood, amniotic fluid and urine of the newborn is dominated by large amounts of conjugated estriol. As reported from this laboratory, there are at least three different conjugated forms of estriol in human body fluids: estriol-3-sulfate; estriol-16 (17?)-glucosiduronate; and another glucosiduronate-like conjugate, perhaps a 3,16-diglucosiduronate (I). A method has now been developed for a separation and estimation of these compounds in cord blood, amniotic fluid and urine of the newborn. In addition, estriol-3-sulfate and estriol-16 (17?)-glucosiduronate have now been isolated and identified in extracts of these body fluids. The pattern of estriol in cord blood is characterized by the preponderance of estriol sulfate; 79 per cent of the total estriol was found in this form. Free estriol was 4 per cent and estriol glucosiduronates were 17 per cent. In contrast, the glucosiduronate fraction constitutes the bulk of estriol both in the amniotic fluid and urine of the newborn. The pattern of estriol in the amniotic fluid was: free, 4 per cent; sulfate, 11 per cent; and glucosiduronate, 85 per cent. The distribution of estriol in the urine of newborn infants was: free, 2 per cent; sulfate, 8 per cent; and glucosiduronate(s), 90 per cent. The principal glucosiduronate obtained from cord blood appears to be the new compound referred to above (I); only a small part of this glucosiduronate fraction was estriol-16 (17?)-glucosiduronate. However, the glucosiduronate fraction obtained from amniotic fluid contained only estriol-16 (17?)-glucosiduronate. The finding that estriol sulfate is the major estrogen in cord blood, whereas estriol glucosiduronate is the principal estrogen of amniotic fluid and urine of the newborn, suggests that transconjugation of estriol takes place in the human fetus and newborn.

The Effect of Experimental Bronchomalacia on Pulmonary Mechanics. G. M. TURINO, R. M. GOLDRING AND L. A. KATZ, New York, N. Y. (introduced by A. P. Fishman).

The role of specific anatomic constituents of lung tissue in the maintenance of normal mechanics of breathing is unknown. The present study concerned the effects of alterations in the rigidity of tracheobronchial cartilage on both the distensibility of the lung and the resistance of the tracheobronchial tree to air flow. For this purpose, cartilage was softened in baby rabbits by the intravenous administration of 2 to 5 ml of a saline extract of crude papain (2 per cent). Pulmonary pressure-volume and pressure-flow relationships were compared before and after papain in unanesthetized animals breathing spontaneously. The techniques involved the simultaneous measurement of intrapleural pressure by direct needle puncture, and tidal volume and rate of air flow by a specially constructed body plethysmograph. An effect of papain on the rigidity of cartilage was manifested clinically by ear drop. An effect on tracheobronchial cartilage was confirmed by 1) abnormal pressure-volume characteristics of isolated tracheal segments and 2) alterations in staining characteristics upon histologic section. Following the administration of papain in 10 test animals, there was no significant difference from the control values either in pulmonary compliance (average 1 ml per cm H_2O, range 0.85 to 1.3) or in airway resistance (average 0.008 cm H_2O per ml per second, range 0.003 to 0.015) during normal breathing. However, the papain-treated animals showed an exaggerated susceptibility to obstruction of the cervical trachea by external compression; this was manifested by abnormally low compliance and high airway resistance. The results indicate that normal rigidity of tracheobronchial cartilage is not essential for patency of large or small pulmonary airways during normal breathing. However, the rigidity of cartilage may prevent airway obstruction during flexion of the neck or extreme variations in intrathoracic pressure.

Human Serum Growth Hormone: Measurements of Concentration and Turnover with a Radioimmunoassay. ROBERT D. UTIGER, MARY L. PARKER AND WILLIAM H. DAUGHADAY,* St. Louis, Mo.

A specific radioimmunoassay of human growth hormone (HGH) has been devised in which HGH competes with HGH-I^{131} for binding by rabbit anti-HGH γ-globulin, similar to the Berson and Yalow insulin assay. Unbound HGH-I^{131} was separated from bound HGH-I^{131} rabbit-antibody complex by goat antirabbit-γ-globulin serum. At present this assay can detect 3 mμg of HGH. Human serum, as well as porcine, ovine and bovine sera, contains a nonspecific inhibitor of HGH-I^{131} precipitation. This can be removed by extracting HGH from serum (Gemzell method). Recovery of millimicrogram amounts of HGH added to serum with each group of unknown sera has varied from 45 to 94 per cent. Correction for this loss has been made. Sera from 21 normal individuals contained less than 60 mμg HGH per ml, while sera from 14 acromegalic patients of varied clinical activity contained 20 to 260 mμg HGH per ml. HGH was injected intravenously in doses of 5 or 10 mg into one hypopituitary and three eupituitary subjects. HGH rapidly disappeared from serum (half-life 22 to 36 minutes) and became undetectable after 5 hours. No HGH was found in the urine. HGH-I^{131} was injected intravenously into two subjects. TCA and immunologically-precipitable I^{131} appeared promptly in the serum and was excreted in the urine. When HGH was given intramuscularly to four subjects, peak serum HGH levels were delayed 3 to 5 hours and were much lower than those obtained following intravenous administration. This radioimmunoassay of extreme sensitivity has demonstrated that HGH circulates in minute concentrations and has a rapid physiologic turnover.

Effect of Feeding Fat to Fasting Subjects. W. P. VANDERLAAN* AND R. R. HALL, La Jolla, Calif.

Although many factors affect the level of circulating fatty acids (FFA), the rise which occurs with fasting is dependent upon the pituitary gland and appears to be a reasonable test for presence of growth hormone. After fasting, the administration of large or small doses of glucose is followed by a prompt decline in FFA and a subsequent return to the previous high level. Albrink and Neuwirth have shown that a fat meal containing only 12 g of protein leads to a similar fall in FFA after 2 to 7 days of fasting but not after overnight fasting. They have interpreted this to indicate that an adaptation to fasting occurs such that a fat meal will lower FFA. We have studied five slightly to grossly obese subjects. After 60 or more hours of fasting, FFA averaged 1,165 μEq per L; 50 g of olive oil was then taken p.o. Two hours later FFA averaged 1,250 μEq per L; 4 hours later, 1,120 μEq per L. Blood sugar levels were unaffected, and ketonemia increased in three subjects. Our previous study suggested that the rise in FFA in the adaptation to fasting was reversed by glucose feeding only so long as blood sugar levels were elevated. This study suggests the specificity of carbohydrate or its precursors in this role.

The Effect of Hydrochlorothiazide on Magnesium Excretion. WARREN E. C. WACKER, Boston, Mass. (introduced by George P. Berry).

The disturbances of neuromuscular function which often accompany the administration of the chlorothiazide drugs have been attributed to the excessive renal loss of potassium. However, in some of these cases, potassium replacement fails to alleviate muscular spasms or cardiac arrhythmias, suggesting additional etiological factors. The intracellular distribution of magnesium, its role in neuromuscular excitation and its capacity to abolish cardiac arrhythmias due to digitalis intoxication, all suggest that this group of diuretic agents may

additionally effect magnesium metabolism. Eight normal persons were maintained on a constant diet throughout the experiment. After a 2-day control period, 200 mg of hydrochlorothiazide was administered on Day 3. The urinary excretion of magnesium during the control period was 180 ± 45 mg per 24 hours; it rose to 280 ± 43 mg during the 24 hours of hydrochlorothiazide administration. The increase in urinary magnesium concentration ranged from 50 to 157 mg per 24 hours and persisted on continued drug administration. Simultaneously, a transient decrease in serum magnesium concentration was observed. Symptomatic magnesium deficiency in man generally occurs as a conditioned deficiency in debilitated patients. These findings now add a drug, hydrochlorothiazide, to the nutritional, hormonal and anatomic conditioning factors already known to bring about the clinical manifestation of magnesium deficiency by an effect on absorption, utilization or excretion of this important intracellular electrolyte. Thus, it seems likely that signs and symptoms arising during the administration of hydrochlorothiazide and which do not respond to therapy with potassium may be alleviated by magnesium replacement therapy. An awareness of this circumstance may further assist to avoid the conditioning of severe magnesium deficiency in patients exhibiting the appropriate metabolic setting.

An Inherited Connective Tissue Syndrome Associated with a Specific Coagulation Defect. Robert L. Wall, Dante Scarpelli and William Molnar, Columbus, Ohio (introduced by Charles A. Doan).

Four generations of an Ohio family have been observed with what appears to be a unique inherited connective tissue disorder. Affected members of this family are of short stature (less than 160 cm). Their long bones are shortened and show multiple gross exostoses. Hemorrhage following surgery, and rarely spontaneously, has occurred from a vascular defect plus a decrease in a specific coagulation component. The vascular defect is a defective elastic fiber showing a total decrease, fragmentation, calcification, and rarely a redundant folding. The coagulation defect is thromboplastin antecedent component. Joint, skin and ocular manifestations have not been observed. The propositus, a 35 year old white female, died of hemorrhage from her pancreatic artery in the absence of pancreatitis 47 days post partum following surgical intervention on four occasions for profuse hemorrhage of pelvic origin. Her surgery was complicated by fragility of tissues to gentle surgical manipulation. Her sister, also in her third decade, bled fatally from her pancreatic artery 9 days post splenectomy for nontraumatic spontaneous rupture of her spleen. The pancreatic arteries of each patient showed decreased, fragmented and calcified elastic fiber. Multiple exostoses have been observed in 5 of 19 adults. Plasma thromboplastin antecedent assays show this deficiency in 18 relatives of the four generations. Genetic transmission is an autosomal dominant. Of the recognized inherited connective tissue syndromes, the present family most closely resembles the Ehler-Danlos syndrome where friability of tissues and unexplained hemorrhages are common, and short stature, while rare, has been described. They differ in the absence of hyperextensibility of joints and hyperelasticity of skin and by the presence of multiple exostoses, plasma thromboplastin antecedent deficiency and deficient elastic fibers of the pancreatic artery, none of which has previously been related to Ehler-Danlos syndrome.

Renal Discrimination between Alkaline Earth Cations. Mackenzie Walser,* Baltimore, Md.

The renal clearances of magnesium, calcium, and radiostrontium are highly correlated under a variety of circumstances, suggesting that alkaline earths may be reabsorbed via a common mechanism. This correlation has been examined in detail in dogs, both in daily urine collections while on varying diets and in clearance experiments in which mannitol or saline was infused. Protein binding differed significantly, averaging 27 per cent for radiostrontium, 33 per cent for magnesium, and 42 per cent for calcium. In dogs on normal or low sodium diets, the major determinant of the clearances of all three cations is the simultaneous clearance of sodium. Salt depletion results in markedly reduced clearances despite mannitol diuresis. Saline infusion or chlormerodrin (Neohydrin) injection increases clearances manyfold. The order of magnitude of the renal clearances, corrected for protein binding, is almost always $Sr > Mg > Ca$. The greatest proportional difference is seen at the lowest excretion rates, and is approximately 6:3:1. At higher rates, the ratios approach 2:1.5:1. The difference between percentage excretion of filtered radiostrontium and filtered calcium increases rapidly at first with increasing calcium clearance, but reaches a maximum of 20 per cent. Although these relationships were not altered by sodium administration, infusion of either calcium or magnesium salts augmented the clearance of the cation infused more than that of the other, or of sodium. On a magnesium-free diet, considerable calcium retention occurred; magnesium conservation was nearly complete, even after calcium infusion. On a calcium-free diet, however, calcium loss persisted unless magnesium was also removed. Under all conditions, radiostrontium clearance followed the clearance of calcium rather than of magnesium.

The Pathogenesis of Experimental Hepatosplenic Schistosomiasis mansoni. Kenneth S. Warren, Bethesda, Md. (introduced by Robert S. Gordon).

The pathogenesis of the liver disease of schistosomiasis has not been elucidated. Conflicting theories hold that hepatosplenic schistosomiasis is due either to the presence in the liver of large numbers of eggs produced by the worms or of dead worms causing large areas of necrosis. Concepts of treatment vary widely depending on which

theory is believed. Those accepting the egg theory believe in complete and rapid destruction of the worms, whereas those supporting the dead worm theory feel that schistosomicidal therapy may be detrimental to the patient. Experimental investigations in the past have relied mainly on histopathological studies, often using inappropriate hosts. Recently the development of hepatosplenic schistosomiasis mansoni (HSSM) in mice has been reported by this laboratory. These animals have hepatosplenomegaly, esophageal varices, ascites, anemia, portal hypertension, sulfobromophthalein retention, hyperammonemia and hyperglobulinemia. Using the development of HSSM as a criterion of significant liver disease the influence of eggs and dead worms was studied both individually and in conjunction. Mice (862) were infected with a number of cercariae sufficient to produce HSSM. Treatment (stibophen) killing almost all of the schistosomes was instituted at 5 weeks when the worms were mature in size and oviposition was just beginning, at 7.5 weeks when many eggs were in the liver, and at 10 weeks when HSSM was completely developed in most of the mice. Untreated mice developed HSSM within 10 weeks. The animals treated at 5 weeks had only a mild transient splenomegaly. Moderate splenomegaly, hepatomegaly and portal hypertension in the group treated at 7.5 weeks rapidly returned to normal, and the mice with HSSM treated at 10 weeks recovered completely within 10 weeks following treatment. In conclusion, dead worms in a number sufficient to cause HSSM, if alive and producing eggs, neither produce nor exacerbate the syndrome.

Abnormalities of Erythrocyte and Plasma Lipids in Acanthocytosis. PETER WAYS, CLAUDE F. REED AND DONALD J. HANAHAN, Seattle, Wash. and Rochester, N. Y. (introduced by Cyrus E. Rubin).

Acanthocytosis is a hereditary syndrome manifested clinically by steatorrhea, progressive neurologic disability, and retinitis pigmentosa. Additional findings include vacuolization of intestinal absorptive cells, low levels of plasma lipids, and absence of β-lipoproteins and chylomicrons. The distinguishing feature is the presence of abnormal "spiny" erythrocytes. In this study such "acanthocytes" from 3 afflicted patients were analyzed; abnormalities in erythrocyte membrane lipids were found. First, despite normal quantities of total red cell phospholipids, their distribution was altered: the lecithin fraction was decreased in all three cases to 21.6, 18.6 and 15 per cent of total prospholipid, respectively (normal, 27 to 32 per cent), with concomitant increases in the sphingomyelin fraction to 28.6, 36.2 and 28.5 per cent (normal, 19 to 24 per cent). In addition, gas chromatography revealed a major abnormality in the profile of fatty acids esterified to pospholipid. Linoleic acid, normally comprising 10 to 12 per cent of the fatty acids present in total red cell phospholipid, averaged only 2.2 per cent in the acanthocyte. Percentages of linoleic acid in individual phospholipid fractions from the acanthocyte showed similar relative decreases when compared with corresponding phospholipids from normal cells. Because the lecithin fraction normally contains 75 per cent of all the linoleic acid found in erythrocyte phospholipid, it appears likely that the alterations in its distribution may be directly related to the reduction in linoleic acid. In plasma, lecithin was decreased both relative to sphingomyelin and in absolute terms. Linoleic acid was consistently decreased in all fractions in which it is normally found. In conjunction with the clinical picture, these abnormalities in erythrocyte and plasma lipid suggest that the composition of intestinal and neural structures may be similarly altered in acanthocytosis, and that the primary defect may reside in the absorption and/or metabolism of linoleic acid.

Influence of Flow Properties of Blood Upon Viscosity-hematocrit Relationships. R. E. WELLS, JR. AND E. W. MERRILL, Boston and Cambridge, Mass. (introduced by E. C. Eppinger).

Numerous investigators have shown that blood viscosity, plotted against hematocrit, rises steeply in an upwardly concave curve. It has also been clearly demonstrated (by relatively few workers) that the viscosity of whole blood is dependent on shear rate, decreasing as shear rate rises. Heretofore, connection between viscosity dependence on hematocrit and viscosity dependence on shear rate has been overlooked. Studies of blood viscosity, by instruments capable of measuring point values of shear stress and of shear rate, have been conducted in which the viscosity of whole blood in absolute values at specific shear rates was plotted against values of hematocrit. At low rates of shear (such as may occur in the arterioles) the viscosity rises very rapidly with increase in hematocrit, while at high rates of shear (such as may occur in the aorta) the relationship of viscosity to hematocrit is considerably more linear. The interpretation of data from studies of dynamics of blood circulation cannot, therefore, assume viscosity to be a single value, nor can changes in hematocrit during a series of observations lead to valid extrapolation of viscosity values, unless the values of viscosity are determined at equal shear rates.

Mechanism of the Anabolic Effect of Testosterone. J. D. WILSON, Dallas, Tex. (introduced by Marvin D. Siperstein).

Despite repeated demonstrations of the marked anabolic effect of testosterone, the mechanism by which this action is mediated is poorly understood. We have therefore studied the effect of testosterone on protein synthesis from lysine-C^{14}, tyrosine-C^{14}, and valine-C^{14} in slices of seminal vesicle and prostate. Testosterone administration markedly enhanced protein synthesis in slices from both normal and castrated rats, reaching a maximum of 10 to 20-fold in 48 to 72 hours. The demonstration of this effect *in vitro* makes possible an

evaluation of the biochemical site of testosterone action. Current concepts of protein biosynthesis are as follows: amino acids (AA) $\xrightarrow{1}$ intracellular AA $\xrightarrow{2}$ adenylates $\xrightarrow{3}$ RNA-AA $\xrightarrow{4}$ miocrosomal-ribonucleoprotein (RNP) $\xrightarrow{5}$ protein. The effect of testosterone on this pathway has been examined at several critical sites. Since intracellular transport of amino acids, and synthesis of new amino acids from acetate-l-C^{14} were both normal in these tissues, testosterone cannot act by regulating the level of intracellular amino acids. Indeed, since the rate of formation of RNA-AA was found to be normal in tissues from testosterone-treated animals, the first three steps of protein synthesis can be excluded as significant sites of testosterone action. These findings suggested that one of the final steps in protein synthesis is accelerated by testosterone. Two distinct types of experiment suggest that the site of this testosterone action is located specifically at step 4. Since step 4 is critically inhibted by reduced incubation temperature, the prevention of this effect by testosterone suggested that its site of action is microsomal-RNP synthesis. Moreover, the ability of testosterone to overcome the specific inhibition of microsomal-RNP synthesis by puromycin is added evidence for this locus of action. These studies constitute strong evidence that testosterone exerts its anabolic action by stimulating a specific site in protein synthesis, the conversion of RNA-AA to microsomal-RNP.

Hypercalcemia Simulating Hyperparathyroidism Induced by XV-2 Carcinoma in Rabbits. J. R. Wilson, H. Merrick and E. R. Woodward, Gainesville, Fla. (introduced by S. P. Martin).

Hypercalcemia has been observed in a variety of human cancers. This investigation was initiated on the possibility that hypercalcemia would be found in the presence of anaplastic carcinoma in the rabbit. Thirteen New Zealand white rabbits received a transplant of XV-2 carcinoma into the anterior thigh muscles. Seven control rabbits were maintained. Blood samples were obtained from 1 to 3 times weekly thereafter and plasma calcium, phosphorus, and blood urea nitrogen were determined. In 11 of the tumor-bearing rabbits there was an increasing degree of hypercalcemia as high as 38 mg% which usually occurred after the third week post implantation. Three animals demonstrated a sustained hypercalcemia with sudden return to control levels within 1 to 2 days prior to death. Plasma phosphorus usually decreased after the third week. Within 1 to 2 days prior to death plasma phosphorus was found at or above control levels in the same animals that demonstrated a rapid decrease in calcium. These same animals showed a progressive increase in BUN as high as 185 mg%. X-ray and histologic study revealed extensive bone resorption, associated with numerous giant cells. Microscopic study of the kidneys revealed marked hyperemia, infiltration with inflammatory cells, and degenerative changes of the proximal tubules with massive proteinaceous exudate. It is concluded that XV-2 carcinoma produces a hyperparathyroid-like state in rabbits, with hypercalcemia, hypophosphatemia, and bony resorption. Three animals apparently developed hypercalcemic nephropathy.

The Acute Hemodynamic Effects of α-Methyl-dopa in Man. William R. Wilson, F. David Fisher and Walter M. Kirkendall, Iowa City, Iowa (introduced by Horace M. Korns).

α-Methyl-3,4-dihydroxy-DL-phenylalanine (α-methyl-dopa) is an inhibitor of several decarboxylases. It has been suggested that its depressor effect in man is related to inhibition of decarboxylation necessary in the reaction, dopa to dopamine. α-Methyl-dopa (0.7 g per m^2) was given intravenously to 5 hypertensives. Observations were made before and at hourly intervals for 3 hours after the drug; identical control observations were made in 3 other hypertensive and 2 normotensive subjects given an equal volume of normal saline. When we compared data from the treated group with those from the controls, the following significant averaged findings were noted: supine intra-arterial mean blood pressure (B.P.) fell 26 mm Hg ($p < 0.02$) in treated hypertensives at 3 hours after the drug, compared with an average drop in the controls of 4 mm Hg ($p < 0.5$); upright B.P. decreased 37 mm Hg ($p < 0.01$) versus 2 mm Hg ($p = 0.9$); pulse rate fell 14 beats per minute ($p < 0.01$) compared with an increase of +3 ($p < 0.4$); cardiac index declined 0.64 L per minute per m^2 ($p < 0.05$) in the treated group, while it increased 0.06 ($p = 0.9$) in the controls; calculated systemic peripheral resistance decreased in 3 of each group. Valsalva overshoot, tiltback overshoot, arterial hematocrit and oxygen saturation altered in the two groups. Mean pulmonary artery pressure was lowered and oxygen consumption was reduced after the drug. Somnolence was usually observed. The pressor effect seen soon after the intravenous administration of guanethidine and bretylium was not noted. These data support the view that acute blood pressure reduction after α-methyl-dopa is caused chiefly by a decrease in cardiac output. The depressor effect, the long latent period before its appearance, and the bradycardia are consistent with the hypothesis that the drug's hemodynamic effect is caused by partial blockade of peripheral synthesis of catecholamines.

The Relation of Insulin to Metabolic Defects in Adipose Tissue Metabolism in Diabetes. Albert I. Winegrad and Walter N. Shaw, Philadelphia, Pa. (introduced by F. D. W. Lukens).

Alterations in the metabolism of tissues from animals with experimental diabetes are usually attributed to a relative or absolute deficiency of insulin, particularly if insulin corrects the impaired glucose metabolism of these tissues. Alloxan diabetes produces several defects

in the metabolism of adipose tissues which can be demonstrated by *in vitro* experiments using rat epididymal fat pads. The response to insulin varies with the specific abnormality. 1) The impaired glucose oxidation to CO_2 and incorporation into long chain fatty acid are promptly stimulated by insulin *in vitro*, and insulin *in vivo* restores them to normal range within 3 hours. 2) Studies of Krebs' cycle activity demonstrate reduced $C^{14}O_2$ production from acetate-1-C^{14}, pyruvate-2-C^{14}, citrate-1,5-C^{14}, α-ketoglutarate-1,5-C^{14}, and fumarate-2,3-C^{14}, and decreased oxygen uptake by adipose tissue incubated with these substrates. Insulin *in vitro*, in the presence of glucose, has no effect on $C^{14}O_2$ production from these substrates or on oxygen uptake. When insulin is injected *in vivo* and adipose tissue removed at intervals and incubated with glucose, 24 hours is required before one observes a significant increase in oxygen uptake. 3) The increased rate of release of free fatty acid (FFA) by adipose tissue from alloxan diabetic rats incubated with glucose and bovine serum albumin is not affected by insulin *in vitro*. Twelve hours is required before injected insulin reduces the rate of FFA release to normal. Thus, in an "insulin sensitive" tissue some of the metabolic defects resulting from alloxan diabetes are not corrected by insulin *in vitro* and respond very slowly to insulin *in vivo*, the time course resembling that described by Renold for liver slices from these animals. These studies suggest that some defects may not result primarily from insulin deficiency but may reflect other hormonal adjustments.

The Metabolism and Distribtion of Angiotensin II-I^{131}. Robert L. Wolf, Julia Pick, Stanley E. Gitlow and Nosrat E. Naftchi, New York, N. Y. (introduced by Milton Mendlowitz).

I^{131}-labeled angiotensin II (ileu-5-angiotensin II and val-5-angiotensin II) has been employed in a quantitative study of the metabolism of this peptide in humans. Angiotensin II-I^{131} was administered intravenously to 11 normotensive subjects; 7 patients with primary benign hypertension (untreated); and to 1 patient with each of the following conditions: primary benign hypertension (treated), primary accelerated hypertension, secondary renal hypertension, pheochromocytoma before and after surgical removal of the tumor; and 1 patient who had previously had acute glomerulonephritis associated with hypertension. The results indicate a mean space of distribution of angiotensin II in the normotensive subjects of 23 L and in the primary untreated hypertensive patients of 26 L. The mean degradation half-time of angiotensin II in the normotensive controls was 10.3 hours compared with 15.8 hours in the primary untreated hypertensive subjects. The mean total exchangeable angiotensin II was calculated to approximate 0.41 μg in the normotensive controls, 0.99 μg in the subjects with primary untreated hypertension and 9.3 μg in one patient with accelerated hypertension. Unlabeled angiotensin II in 50 μg intravenous doses did not alter the degradation rate of angiotensin II-I^{131} in normotensive and hypertensive human subjects. The degradation of angiotensin II-I^{131} in these experiments, therefore, followed first order kinetics. The angiotensin II degradation rate of the patient who previously had hypertension associated with acute glomerulonephritis was normal. The primary hypertensive subject treated with hydrochlorothiazide with a normal blood pressure, the patient with secondary renal hypertension, and the subject with the pheochromocytoma both before and after surgical removal of the tumor had an angiotensin II degradation rate within the range of untreated patients with primary hypertension. The slow rate of degradation of angiotensin II-I^{131} in the circulation of subjects with untreated primary hypertension is partially if not completely responsible for the increased concentration of angiotensin II in the body fluids of these patients.

Genetic Control of the Ability of Human Blood to Neutralize Angiotensin and its Relationship to Essential Hypertension. J. Edwin Wood,* Augusta, Ga.

Variation in capacity of whole blood to neutralize angiotensin was investigated regarding its role in essential hypertension. Blood (30 ml), 45.0 μg of synthetic angiotensin (Ciba) and 3 mg of heparin were incubated (37° C) for 10 minutes, then infused into the donor at the rate of 1.91 ml per minute. The subject could not observe the onset of infusion. Arterial pressure was measured (auscultatory) by an observer unapprised of the blood pressure of the subject's parents. An increase of diastolic pressure of 10 mm Hg or more during infusion constituted a positive test. The result was not revealed to another observer who measured resting blood pressure of the subject's parents at their home. Parents (ages 41 to 69) with diastolic pressures of 90 mm Hg or more were considered hypertensive (35 per cent). Seventy-one freshman medical students were tested. Twenty-eight families were composed of 2 normotensive parents with 27 offspring having a negative test. Thirty-six families were composed of 1 hypertensive and 1 normotensive parent with 18 offspring having a positive test (predicted 20, $q = 0.80$) and 18 a negative test (predicted 16). Seven families were composed of 2 hypertensive parents with 4 offspring having a positive test and 3 a negative test. All parents in two of these families were tested and were positive. Five offspring demonstrated both positive and negative tests in each family. Ten of 12 patients thought to have essential hypertension had positive tests. Infusion of angiotensin with saline did not separate student subjects with positive and negative tests. Plasma hemoglobin concentration of infused bloods was negligible. These findings imply that a simple dominant gene may lead to essential hypertension through the characteristic of relative inability to destroy naturally occurring angiotensin.

Failure of Formiminoglutamic Acid (FiGlu) Excretion to Distinguish Vitamin B_{12} Deficiency from Nutritional Folic Acid Deficiency. RALPH ZALUSKY AND VICTOR HERBERT, Boston, Mass. (introduced by William B. Castle).

It has been suggested that measurement of FiGlu excretion in the urine following an oral histidine load distinguishes patients with folic acid deficiency from those with vitamin B_{12} deficiency. Studies in rats and chicks have shown that formiminoglutamicaciduria occurred with deficiency of either vitamin. To evaluate this subject in man, 12 patients with megaloblastic anemia were studied. FiGlu was measured qualitatively by paper electrophoresis at 400 v, and quantitatively by the enzymatic-spectrophotometric method and by the colorimetric alkaline ferricyanide nitroprusside reaction. Six patients had low serum vitamin B_{12} levels measured by *Euglena gracilis* (12 to 42 $\mu\mu$g per ml) and normal serum folic acid activity measured by *Lactobacillus casei* (7 to 21.5 mμg per ml). Six patients had low normal to high serum vitamin B_{12} levels (107 to 1,288 $\mu\mu$g per ml) and low serum folic acid activity (<1 to 1.5 mμg per ml). Two of the vitamin B_{12}-deficient patients and all of the folic acid-deficient patients had abnormal FiGlu excretion following 5 to 20 g L-histidine orally. Of the two vitamin B_{12}-deficient patients with abnormal FiGlu excretion, 0.82 mg folic acid intravenously in one, and 15 mg folic acid intramuscularly daily for 2 days in the other, resulted in an increase rather than decrease of FiGlu excretion. Vitamin B_{12} therapy, 5 μg daily for 7 days in one case, and 30 μg daily for 15 days in the other, led to a marked diminution of FiGlu excretion within 10 days. These results indicate that while formiminoglutamicaciduria after histidine-loading occurs in nutritional folic acid deficiency, it may also occur in vitamin B_{12} deficiency. It is suggested that vitamin B_{12} may be required for adequate utilization of folic acid.

The Influence of Thyroid Hormone on Cortisol Production and Metabolism. BARNETT ZUMOFF, LEON HELLMAN,* H. L. BRADLOW, D. K. FUKUSHIMA AND T. F. GALLAGHER, New York, N. Y.

Steroid isolation studies in myxedematous, euthyroid and hyperthyroid subjects have demonstrated that profound alterations in the metabolism and production of cortisol (F) occur in direct relation to the level of thyroid function. 1) Following administration of thyroid hormone or in spontaneous hyperthyroidism there is increased conversion of endogenous F to its 11-ketonic metabolites (tetrahydrocortisone, cortolone, 11-ketoetiocholanolone). In contrast, hypothyroidism or treatment of hyperthyroidism favors the transformation of F to the 11-hydroxy metabolite, tetrahydrocortisol. These findings are the result of changes in peripheral metabolism as demonstrated by parallel tracer studies with cortisol-4-C^{14}. 2) In hyperthyroidism, the total production of F is markedly increased, frequently to levels observed in Cushing's syndrome. Reduction in F secretion is found in hypothyroidism. 3) This modification of the route of metabolism of the carbon-11 position in the corticoid series is a new effect of thyroid hormone and is to be compared with the previous demonstration that thyroid favors the formation of 5-α reduction products (androsterone) in the androgen series. 4) The increased F production in hyperthyroidism appears to be dependent on the augmented formation of the 11-ketonic metabolites; i.e., these represent increased metabolic inactivation of F. The subsequently diminished availability of F results in operation of a negative feedback mechanism for the stimulation of pituitary ACTH production until an additional quantity of F is produced, sufficient to operate the pituitary suppressive mechanism. This is interpreted as evidence for the feedback control of a tropic hormone by alterations in the metabolism of the secretory product of the target organ. 5) This thyroid-induced metabolic acceleration of F inactivation accounts for the failure to develop a typical Cushing's syndrome in hyperthyroidism in spite of the suitably elevated F production and, probably, for the finding of signs and symptoms of adrenal insufficiency in thyroid storm.

CLINICAL SCIENCE

Editorial Board

for the Medical Research Society

G. M. BULL, *Chairman*, A. C. DORNHORST, A. D. M. GREENFIELD, E. J. M. CAMPBELL

for the Biochemical Society

N. F. MACLAGAN, *Deputy Chairman*, C. H. GRAY, N. H. MARTIN, F. T. G. PRUNTY

Volume 20, Part 2.

The transfer of bromsulphthalein from the plasma to the bile in man. By G. BARBER-RILEY, A. E. GOETZEE, T. G. RICHARDS and J. Y. THOMPSON.

Metabolism of 131_I labelled human albumin. By S. COHEN, T. FREEMAN and A. S. MCFARLANE.

Respiratory adaptation to exercise in anxious patients. By A. G. MEZEY and A. J. COPPEN.

Vitamin B_{12} metabolism in polycythaemia vera. By I. W. DELAMORE.

The localization of 14C labelled vitamin D_2 in the nephron. By E. KODICEK, E. M. DARMADY and F. STRANACK.

Pyruvate metabolism in Wilson's disease. By J. M. WALSHE.

Assessment of anaemia in clinical practice. By T. S. LEE and G. R. WADSWORTH.

Changes in blood flow and temperature of muscle in the chilled limb of the rabbit. By A. SAYEN and C. BOLAND.

The ability of man to detect added elastic loads to breathing. By E. J. M. CAMPBELL, S. FREEDMAN, P. S. SMITH and M. E. TAYLOR.

The effect of minute and increasing doses of aldosterone with saline loading on young and older mice. A means of detecting small amounts of aldosterone. By D. B. CHEEK and A. B. HOLT.

Blood pressure and body build in men in tropical and temperate Australia. By A. E. DOYLE and R. R. H. LOVELL.

A mechanism for the action of unsaturated fat in reducing the serum cholesterol. By T. PILKINGTON, K. A. HODD and B. LEWIS.

The fatty acid patterns of human serum in health. By T. D. V. LAWRIE, S. G. MCALPINE, R. PIRRIE and B. M. RIFKIND.

The effect of dilution on the titratable acid in urine and acidified phosphate buffer solutions and the correction for this effect in the determination of the rate of elimination of hydrogen ions from the body by renal tubules. By D. M. NUTBOURNE.

Volume 20, Part 3.

An assessment of erythrocyte migration times and serum cholesterol levels as indices in myocardial infarction. By D. F. DAVIES and A. CLARK.

The mechanism of the vasodilator action of reserpine in man. By V. J. PARKS, A. G. SANDISON, S. L. SKINNER and R. F. WHELAN.

Intracellular magnesium deficiency in man. By I. MACINTYRE, S. HANNA, C. C. BOOTH and A. E. READ.

The antihypertensive endocrine function of the kidney. By T. TÓTH and J. BARTFAI.

The use of ^{51}Cr and ^{59}Fe as red cell labels to determine the fate of normal erythrocytes in the rabbit. By N. C. HUGHES JONES.

The use of ^{51}Cr and ^{59}Fe as red cell labels to determine the fate of normal erythrocytes in the rat. By N. C. HUGHES JONES and B. CHENEY.

Removal of radiostrontium in man by orally administered ammonium chloride two weeks after exposure: the effect of low and high calcium intake. By H. SPENCER and J. SAMACHSON.

Quantitative measures of deep somatic pain: preliminary study with hypertonic saline. By B. B. WOLFF, J. L. POTTER, W. L. VERMEER and C. MCEWEN.

The production of lipolytic activity in the human forearm in response to heparin. By J. R. FRASER, R. R. LOVELL and P. J. NESTEL.

The oxalate content of human plasma. By C. J. CRAWHALL and R. W. WATTS.

Evidence for two vascular pathways in skeletal muscle. By T. BARLOW, A. L. HAIGH and D. N. WALDER.

Glycine metabolism in acute porphyria. By F. F. RICHARDS and J. J. SCOTT.

The effect of the intravenous infusion of noradrenaline on splanchnic blood flow in haemorrhagic shock. By H. B. TORRANCE.

Effect of chlorothiazide on blood pressure and blood flow at rest and on exercise in patients with arterial hypertension. By E. VARNAUSKAS, G. CRAMER, R. MALMCRONA and L. WERKÖ.

Subscription Rate: $13.50 per volume of three parts. (*$27.00 per year*)

orders may be placed with your bookseller, or sent direct to the publishers

CAMBRIDGE UNIVERSITY PRESS

32 East 57th Street, New York 22, N. Y. Bentley House, 200 Euston Road, London, N.W. 1

The Journal of Clinical Investigation
333 Cedar Street New Haven 11, Conn.

COMPLETE VOLUMES
OF
BACK ISSUES

Vol. 27 (1948) six issues and one supplement	10.00
Vol. 28 (1949) six issues and two supplements	10.00
Vol. 29 (1950) twelve issues	10.00
Vol. 30 (1951) twelve issues and one supplement	12.00
Vol. 31 (1952) twelve issues	12.00
Vol. 32 (1953) twelve issues	12.00
Vol. 33 (1954) twelve issues	12.00
Vol. 36 (1957) twelve issues and two supplements	15.00
Vol. 37 (1958) twelve issues	15.00
Index, Vols. 1–17, inc.	2.00

Order While Available

J.C.I. SUPPLEMENTS
Order while still available

Symposium on Malaria May, 1948, Part II	$2.00
Symposium on Antibiotics September, 1949, Part I	$2.00
Symposium on Radioactive Isotopes November, 1949, Part I	$2.00
Treatment of Acute Infectious Hepatitis July, 1955, Part II	$1.50
The Renal Lesion in Epidemic Hemorrhagic Fever January, 1957, Part II	$1.50
Metabolic Studies in Paralytic Acute Anterior Poliomyelitis June, 1957, Part II	$1.50

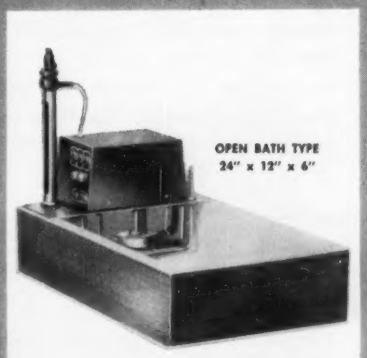
* * * * *

Studies on Influenza in the 1957–1958 Pandemic January, 1959, Part II	$1.50
History of the A.S.C.I. October, 1959, Pt. II	$1.50

Order through the Business Office of
THE JOURNAL OF CLINICAL INVESTIGATION
333 Cedar Street New Haven 11, Conn.

HETO
CONTROL TO 1/10° C
WATER THERMOSTATS
Individual, compact and flexible thermostats are a requirement in present day laboratories. HETO thermostats offer compactness and reliability in several styles and pump capacities; noiseless, radiation-free motors . . . and dual capacity controlled heater . . . cooling coils . . . all stainless tanks and fittings . . . and water flow control. Ideal for any temperature control application.

CLOSED TANK 14" x 5" x 2½"


OPEN BATH TYPE
24" x 12" x 6"

Other sizes and types available.
Write for Complete Heto Catalogue.

SOLD AND SERVICED IN U.S.A. BY
THE LONDON COMPANY
(Formerly Welwyn International Inc.)
3355 Edgecliff Terrace CLEVELAND 11, OHIO
In Canada: Bach-Simpson Ltd.

REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

May, 1961

Vol. VI, No. 5

Editorial

Etiocholanolone, Steroides Pyrogenes et "Maladie Periodique. E.-E. BAULIEU.

Original Articles

Analyse Immunologique du Serum Humain Normal apres Electrophorese en Gel d'Amidon. M. DEBRAY-SACHS, B. ANTOINE et J. M. FINE.

Etude Immuno-Electrophoretique du Serum des Prematures. M. FOUCAUT et M. GOUDEMAND.

Etude Quantitative des Gamma-Globulines Seriques chez des Souris Irradiees et Restaurees par des Cellules Hematopoietiques Isologues ou Homologues. J.-L. AMIEL, G. MATHÉ et M. PAYS.

Etude Biochimique d'un Cas de Carcinoidose. A. CRASTES de PAULET, O. FLANDRE et I. COHEN.

Original Notes

Modification du Comportement Antigenique de l'Albumine du Foie au Cours du Developpement de la Tumeur de Walker chez le Rat. D. DUFOUR, DINH BAO LINH, M. DEMERS et P. LINDSAY.

Le Transfert des Grosses Molecules du Sang dans la Bile et le Lymphe Hepatique chez le Cobaye. Etude par le Polyvinylpyrrolidone L^{131}. J. CHENDEROVITCH, E. PHOCAS, S. TROUPEL, H. RENAULT et J. CAROLI.

Influence des Divers Anticoagulants sur la Mesure du pH du Plasma Sanguin. C. SAUNIER, TUFIK-SIMAO et TH. COLAS.

Les Variations avec l'Age de la Densite Osseuse chez le Rat. F. BOURLIÈRE et J. DRY.

Dosage Turbidimetrique des Lipoproteines Seriques de Faible Densite apres Floculation Selective par l'Acide Phosphotungstique a pH Neutre en Presence d'Une Concentration Saline Elevee. M. BURSTEIN et M. BERLINSKI.

General Review

Mecanismes et Syndromes en Immuno-Pathologie. G. VOISIN.

Technical Note

Test de la Niacine et Identification des Mycobacteries. (Valeur de la Methode.) J. VIALLIER et J. AUGAGNEUR.

Subscription rate, $19.00

Special rate for interns and residents, $13.00

EDITIONS MEDICALES FLAMMARION, 22, rue de Vaugirard, PARIS 6ème. France.

AUTOMATION IN

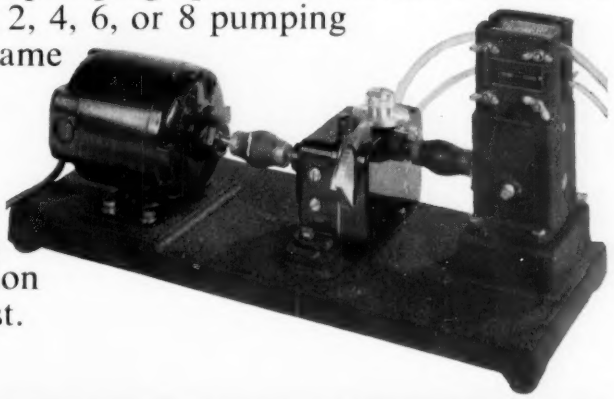
COLUMN CHROMATOGRAPHY

with the SIGMAMOTOR PUMP

Vernier Adjustment Insures Accuracy at Low Flows

Various column chromatography metering operations are simplified by the use of a peristaltic type constant volume pump, such as the Sigmamotor Model T-8. A typical installation combines the Sigmamotor pump with solenoid actuated valves and timing equipment to deliver elutant buffers into ion exchange columns on a reproducible schedule. The T-8 pump is equipped with vernier for accurate flow adjustment.

Single Sigmamotor units can be provided to handle 1, 2, 3, or 4 simultaneous pumping operations. Double units are available to handle 2, 4, 6, or 8 pumping operations at the same time. Capacities from 1 cc to 250 cc/minute are obtained by the vernier adjustment. Complete information available on request.



SIGMAMOTOR Inc.

91 NORTH MAIN STREET • MIDDLEPORT, NEW YORK

NEW VERSATILITY IN Liquid Scintillation Counting

With the Packard Tri-Carb® Spectrometer

Now you can count assortments of carbon-14 and tritium samples interchangeably without readjusting controls . . . perform double labeling at the true balance point for each isotope . . . or do both. In addition, the new Tri-Carb Spectrometer with its expander-amplifier circuitry can be used for dual-channel gamma counting merely by adding a well detector. This versatility is a Packard exclusive—made possible by two separate channels of pulse-height analysis with individually variable amplifiers and four adjustable discriminator levels.

Other features are all-transistorized circuitry and convenient mounting of all controls at eye level above the freezer. Width of the instrument is reduced to a space-saving 3½ feet.

Three models in various combinations offer a total of nine liquid scintillation counting systems—refrigerated or non-refrigerated . . . for automatic or semi-automatic operation. From this selection you can readily meet the requirements of your budget and provide for present and future needs. You'll get the satisfaction and performance which only a Tri-Carb Spectrometer—with its world-wide reputation for accuracy and reliability—can provide. Call your Packard representative or write for Bulletin AD-1002.

INSTRUMENTS FOR RADIOACTIVITY MEASUREMENT AND CHROMATOGRAPHY

Packard

BRANCH OFFICES
CHICAGO • ALBUQUERQUE • ATLANTA • BOSTON
LOS ANGELES • NEW YORK • PHILADELPHIA • PITTSBURGH
SAN FRANCISCO • WASHINGTON, D.C. • ZURICH • PARIS

PACKARD INSTRUMENT COMPANY, INC.
LA GRANGE 53, ILLINOIS

LANCASTER PRESS, INC., LANCASTER, PA.